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14. ABSTRACT I received approval of the DOD-HSRRB (Log No. A-12517) around 20th April, 2005 and began the study without further delay. We conducted portions of task 1, 2, 3, 5 and 6 studies on radical prostatectomy (RP) and biopsy tissue samples and collected clinical data from the same white and African American men. The Elisa assay, part of task 4, laser capture microdisection (LCM) in this task is in progress. Prostate cancer (PC), and benign prostatic hyperplasia (BPH) as control samples, were collected at the VA Medical Center and Virginia Urology Center. Our study has resulted in two manuscripts, 1. "Prognostic Value of the Cathepsin B to Stefin A Ratio in Prostate Needle Biopsies" and 2. "Characterization of Prostate Cancer in African American Men by Cathepsin B and Stefin A." Since all co-authors have not reviewed the manuscripts, we are enclosing drafts prior to their submission to peer-reviewed journals. Study 1. Our objective was to define characteristics of cancer cells in low volume (about 10% in 1 or 2 biopsy cores) and high volume (about 50% in 1 or 2 cores) Gleason score 6 tumor biopsies using cathepsin B (CB) and stefin A (SA), immunohistochemistry (IHC) and quantitative image analysis. We evaluated biopsies with the Gleason score 6 tumors in 65 patients and post-RP specimens. Cancer had relapsed in three patients, as indicated by rising serum total prostate specific antigen (PSA) levels in less than five years even though the post-RP pathology report did not detect cancer cell invasion to prostate margin/capsule, seminal vesicle, and/or pelvic lymph node. Ratios of CB to SA were significantly higher in cancer than in BPH. Study 2. In the Gleason score 6 and 7 tumors, the ratios of CB to SA were not significantly different in biopsies from those in prostectomies, indicating the reliability of immunostaining in small tissue sections. This finding allows testing the utility of these markers in biopsy samples prior to selection of specific treatment and design of prospective studies. Biopsies showed significantly higher levels of CB to SA ratios than BPH.

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Annual Report
March 1, 2005-February 28, 2006
Award Number W81XWH-04-1-0245

Title: Protocol "Prediction of Aggressive Human Prostate Cancer by Cathepsin B,"

Introduction: I received approval of the DOD-HSRRB (Log No. A-12517) around 20th April, 2005 and began the study without further delay. We are enclosing two drafts of the manuscripts and presenting our study #1 and study #2 each section designed by the DOD. All co-authors have not reviewed the manuscripts and the drafts will, undoubtedly, be revised possibly with newer data prior to their submission to peer-reviewed journals.

Study #1. We have previously demonstrated a significant positive association between pelvic lymph node metastases and increased expression of cathepsin B (CB) relative to its inhibitor stefin A (SA) in patients with prostate cancer (PC) who underwent radical prostatectomy (RP). Our objective in this study was to evaluate these markers in prostate needle biopsies showing Gleason histological score 6 tumors. Formalin-fixed, paraffin embedded specimens from 65 patients were subjected to immunohistochemical (IHC) localization of CB (mouse anti-human CB IgG) and SA (goat anti-human SA IgG). Immunostaining was quantified using a computer-based image analysis system equipped with Metamorph software. Data were analyzed using univariate and multivariate techniques, and with Student's t-test ($p<0.05$) for statistical significance.

Study #2. In the era of serum total prostate specific antigen (PSA) measurements, many patients are diagnosed with prostate adenocarcinoma (PC) because of few cancerous glands and invasive cells in 1 or 2 needle biopsy cores out of 6 to 12 cores. Because PC is complex and heterogeneous disease, treatment selection based on limited pre-treatment clinical data is often inaccurate. The inability to select aggressive cancer within each Gleason score/grade greatly affects survival and quality of life of patients. Our objective was to assess the distribution of molecular markers, cathepsin B (CB) and stefin (cystatin) A (SA), in low and high volume tumors in the same patients. We evaluated formalin-fixed, paraffin-embedded prostate biopsy and RP tissue samples from 28 patients and localized CB (mouse anti-human CB IgG) and stefin A (goat anti-human stefin A IgG) by immunohistochemical (IHC) methods. Immunostaining in prostate epithelial cells of cancerous and benign prostatic hyperplasia (BPH) areas was quantified using a computer-based image analysis system equipped with Metamorph software. Data were analyzed using Student's t-test ($p<0.05$) for statistical significance.

Body of the Report: We conducted portions of task 1, 2, 3, 5 and 6 studies on radical prostatectomy (RP) and biopsy tissue samples and collected clinical data from the same white and African American PC patients. The Elisa assay, part of task 4, laser capture microdisection (LCM) in this task is in progress. Prostate cancer, and benign prostatic hyperplasia (BPH) as control samples, are being collected at the VA Medical Center and from the Virginia Urology Center.

Study #1. **Profile of Prostate Cancer Patients:** The age of PC patients at the initial diagnosis ranged from 47 to 73 years (mean ± standard error of the mean (SEM) of 62.7 ± 0.8) (Table 1). All patients had Gleason score 6 tumors both in prostate needle biopsies as well as RP specimens. The regional pelvic lymph nodes were negative for cancer cells in 59 patients (59/65, 90.8%) and node status was unknown in six cases (6/65, 9.2%). None of the patients had evidence of distant metastases based on bone scan and/or clinical data. The clinical stages ranged from T1c to T3b with the majority of patients showing stage T2c tumors (34/65, 52.3%), T2a (14/65, 21.5%), and T2b (13/65, 20%) (Table 1). The number of cases with T1c, T3a and T3b stages was limited. Pre-surgery serum total PSA ranged from 1.25 to 20.0 ng/ml (mean of 6.7 ± 0.5) (Table 1, Fig.1). Pre-surgery PSA levels less than 9ng/ml were associated with stage T2a, while T2b and T2c stages showed considerable variations. In our series, 9/65 (13.8%) cases had 10 ng/ml and higher serum total PSA levels (Fig.

1). Fifty-five (55/65, 84.6%) patients had pre-RP serum PSA levels less than 10ng/ml and the remaining patient had unknown levels. The post-surgery PSA levels were taken between 2 months and 7.5 years after the RP (mean 4.9 ± 1.35 years) and ranged from 0.0 to 0.62ng/ml (mean 0.02 ± 0.01). Three patients with pre-surgery PSA levels of 2.8, 5.9, and 3.64 ng/ml, showed evidence of biochemical recurrence of PC after 4.35, 5.03, and 4.00 years, respectively, in post-surgery clinical data (Table 3). One patient had a post-surgery PSA level above 0.1ng/ml, while the other two patients had post-surgery PSA levels above 0.2ng/ml. PSA levels had not increased in the remaining 62 patients.

Immunostaining of Cathepsin B and Stefin A in Benign Prostatic Hyperplasia Glands: CB and SA immunostaining was present predominantly in the cytoplasm of basal cells and some cuboidal/columnar cells of BPH glands (Fig. 5 A, B). Immunostaining of CB ranged from 1.48 to 5.43, with a mean of 3.14 ± 0.13 (Table 2). Likewise, immunostaining of SA ranged from 1.09 to 4.41, with a mean of 2.70 ± 0.09. The ratios of CB to SA ranged from 0.62 to 2.94, with a mean of 1.21 ± 0.05 (Table 2).

Immunostaining of Cathepsin B and Stefin A in PIN Glands: In PIN glands, the two markers localized in the basal cells (Fig.5 C, D). Immunostaining of CB ranged from 1.39 to 6.40, with a mean of 3.34 ± 0.23. Likewise, SA localization ranged from 1.03 to 3.96, with a mean of 2.39 ± 0.16. The ratios of CB to SA ranged from 0.47 to 4.5, with a mean of 1.65 ± 0.19 (Table 2).

Immunostaining of Cathepsin B and Stefin A in Prostate Cancer: CB and SA localized to the cancer cells in the malignant glands (Fig.5 E-H). The distribution of CB and SA protein reaction products showed considerable variations within Gleason score 6 tumors that are considered histologically and morphologically similar. We have previously noted this phenomenon in RP specimens (15). Immunostaining of CB ranged from 1.43 to 5.81, with a mean of 3.26 ± 0.12. SA localization ranged from 0.12 to 3.11, with a mean of 1.02 ± 0.09. The ratios of CB to SA ranged from 0.85 to 19.54, with a mean of 4.89 ± 0.48 (Table 2) (Fig. 5 E-H).

Analysis of Cathepsin B and Stefin A Immunostaining Data: The immunostaining pattern of BPH glands was used as a control for data analysis. Differences in immunostaining of CB alone were not statistically significant in BPH, PIN and cancer, but SA alone was significantly lower in PC ($P < 0.0001$) when compared to BPH glands and PIN (Table 2). Ratios of CB to SA were significantly higher in cancer when compared to BPH glands and PIN ($P<0.0001$) and also in BPH glands compared to PIN ($P=0.036$) (Table 2) (Fig. 2). Three patients with rising PSA levels (biochemical recurrence) had a clinical stage of T2c, T2c, and T2a and CB to SA ratios of 2.66, 4.92, and 11.46 in cancer areas, respectively (Table 3). In contrast, CB to SA ratios in BPH glands of two of the cases were 1.42 and 1.16 respectively. Thus, ratios of CB to SA were significantly lower in the BPH glands of these two cases when compared to malignant foci. The CB to SA ratio in the BPH glands in the third case with biochemical recurrence could not be computed due to paucity of BPH glands in the specimen.

Cathepsin B and Stefin A Ratios in Relation to Clinical Stages and PSA Levels: Our data showed that higher CB to SA ratios were predominantly associated with T2a, T2b and T2c clinical stages, and a few cases had T1c, T3a and T3b stages (Fig. 3). The average ratios of CB to SA showed an inverse relationship to T2a to T3b clinical stages (Fig. 4). Likewise, patients with T2a, T2b, and T2c stages were associated with CB to SA ratios that ranged from 0.85 to 19.54, with a mean of 5.04 ± 0.50. The single case of T1c did not show the above pattern. Patients with T2a, T2b and T2c clinical stages were associated with variable pre-RP serum PSA levels that ranged from 1.25 to 20, with a mean of 6.56 ± 0.47 (Fig. 1). Nine patients (9/65, 13.8%) had PSA levels ≥ 10 ng/ml and these were associated with T2b, T2c, and T3a clinical stages (Figs. 1, 4). Fifty-five (55/65, 84.6%) patients had pre-RP serum PSA levels less than 10ng/ml and the remaining patient had unknown levels. Tables and figures are attached in the Appendix section.

Discussion: In the current study, we studied the predictive value of CB to SA ratios in prostate needle biopsies. Cathepsin B, a cysteine protease, is involved in the degradation of basement membrane (BM) and extracellular matrix (ECM) proteins and is associated with progression in PC and other solid tumors (18-20, 22, 23). Since it is regulated by its endogenous inhibitor, stefin A, ratios of CB to SA provide better prediction of human PC progression than CB or SA alone in biological compartments. Our analysis of prostate biopsies indicated significantly higher ($P<0.0001$) ratios of CB to SA in malignant glands when compared to BPH and PIN gland areas in the same case. Heterogeneity was found in the expression ratios of CB to SA (mean 4.89 ± 0.48; range 0.85 to 19.54). This is similar to the pattern previously found by us in RP specimens (15). Three patients in our series had biochemical failure shown by serum PSA levels rising to 0.1ng/ml or higher (Table 3). Two of these three cases had positive resection margins in RP specimens. Two of the three cases were given external beam radiation and had undetectable PSA at last follow-up. The third patient moved and was lost to further follow-up. Ratios of CB to SA ratios were 2.66, 4.92, and 11.46. Due to the small number of adverse events in our series, statistical correlation between elevated CB to SA ratio and the risk of biochemical recurrence was not attempted. Nine of 65 (13.8%) cases showed CB to SA ratio greater than 10. Only one of these 9 showed biochemical recurrence (mentioned above); the remaining 8 cases showed no evidence of disease at last follow-up. The number of cases in this series is quite small and only 3 of 65 (4.6%) patients showed evidence of biochemical recurrence. The mean follow-up period in our study was 6.68 years. Many of the cases in our study with elevated CB to SA ratio have been followed for less than 5 years. Long-term follow-up of the entire group is being followed.

Study #2 **Profile of Prostate Cancer Patients:** The age of patients at prostatectomy ranged between 48 and 74 (mean 62.24 ± 1.31). The distribution of Gleason histologic scores ranged from 6 to 8 in biopsy and prostatectomy cases (Tables 1, 2). In biopsy patients, pre-surgery serum total PSA levels ranged from 0.92 to 22.50 (mean 7.46 ± 1.34 SEM), with no PSA data in 6 patients (Table 1). In 15/28 (53.57%) prostatectomy cases, post-surgery serum total PSA levels were >0.2ng/ml indicating biochemical failure and the remaining 13/28 (46.43%) had PSA levels of $\leq$0.2ng/ml. Clinical stages ranged from normal to T3a, including unknown stages in 14 biopsy samples (Table 1). Clinical stages ranged from T2a to T3c, N1-N-3 and unknown in prostatectomy cases (Table 2). We defined aggressive PC by the presence of cancer cells in seminal vesicles and/or pelvic lymph nodes. These characteristics of cancer cells were applied to our analysis of markers (Tables 2, 4). Post-prostatectomy data showed 28.57% (8/28) had developed aggressive prostate cancer, 50% (14/28) had not developed aggressive cancer and aggressiveness was unknown in 21.43% (6/28) of cases (Table 2).

Immunostaining of Cathepsin B and Stefin A in BPH Glands: CB and SA protein immunostaining were present predominantly in basal cells and some cuboidal/columnar cells of BPH glands of both biopsy and prostatectomy tissue sections. Immunostaining for CB alone and SA were similar or had higher SA in biopsy and prostatectomy BPH cases (Fig. 1a, b).

Immunostaining of Cathepsin B and Stefin A in Prostate Cancer: Immunostaining of CB and SA proteins were observed in cuboidal/columnar and isolated cancer cells in biopsy tissue sections (Fig.3). The distribution of reaction products in CB alone and SA alone showed variations between and within Gleason score tumors in biopsy and prostatectomy samples (Fig. 3). In contrast, SA alone was lower than CB in biopsy and prostatectomy cases of Gleason score 6 tumors (Fig. 1a). Stefin A alone was lower in biopsy when compared to CB in cancer and essentially similar in prostatectomy samples of Gleason score 7 tumors (Fig. 1b). Ratios of CB to SA in biopsy cases were significantly elevated in Gleason score 6 ($p=0.02$) and score 7 ($p=0.004$) tumors (Fig. 2a, b). In contrast, ratios of CB to SA in BPH and cancer of Gleason score 6 prostatectomy samples were not significant ($p=0.30$) when compared to BPH (Fig. 2a), but CB to SA ratios were significant ($p=0.05$) in Gleason score 7 prostatectomy samples (Fig. 2b).

Analysis of Cathepsin B and Stefin A in BPH and Cancer: Since BPH is not an invasive tumor, it was used as a control for comparing with malignant tumors. Using our criteria of defining aggressive prostate cancer, we found 8 aggressive cancer, 14 non-aggressive cancer and status of 6 unknown in prostatectomy cases (Table 2). CB to SA ratios that did not follow the immunostaining patterns were considered as outliers in both

biopsy and prostatectomy cases (Tables 3, 4). These cases are planned to be repeated with a new set of sections.

Key Research Accomplishments:

Study #1

- Ratios of CB to SA were significantly higher in cancer when compared to BPH glands (P<0.0001).
- Three patients with rising PSA levels (biochemical recurrence) had a clinical stage of T2c, T2c, and T2a and CB to SA ratios of 2.66, 4.92, and 11.46 in cancer areas, respectively.
- The average ratios of CB to SA showed an inverse relationship to T2a to T3b clinical stages.
- The number of cases in this series is quite small and only 3 of 65 (4.6%) patients showed evidence of biochemical recurrence. The mean follow-up period in our study was 6.68 years.

Study #2

- The distribution of CB and SA reaction products showed variations between and within Gleason score tumors in both biopsy and prostatectomy tissue sections.
- Post-RP surgery serum total PSA levels were >0.2ng/ml in 15 (53.57%) patients indicated that more African American men had biochemical failures than patients with $\leq$ 0.2 ng/ml in 13 (46.43%).
- Ratios of CB to SA in biopsy cases were significantly elevated in Gleason score 6 (p=0.02) and score 7 (p=0.004) tumors.
- Our interim analysis showed that biopsy and prostatectomy sample sizes showing several Gleason scores were too small for a definitive conclusion without addition of new data.

Reportable Outcomes:

Our study has resulted in two manuscripts that are yet to be submitted and are under review by other coauthors:

Study # 1. Prognostic Value of the Cathepsin B to Stefin A Ratio in Prostate Needle Biopsies

Study #2. Characterization of Prostate Cancer in African American Men by Cathepsin B and Stefin A.

Conclusion:

Study #1. CB immunostaining alone was not statistically different in cancer, but SA alone was significantly lower in PC (P< 0.0001) than in BPH glands. Ratios of CB to SA were significantly higher in cancer when compared to BPH (P<0.0001). Ratios of CB to SA in turn were significantly higher in cancer than in PIN (P<0.0001). Our data show that PC is a heterogenous disease within a single Gleason grade with respect to CB to SA ratios. The average ratios of CB to SA showed an inverse relationship to T2a to T3b clinical stages.

Study #. In biopsy patients, pre-surgery serum total PSA levels ranged from 0.92 to 22.50 (mean 7.46±1.34 SEM). In 15/28 (53.57%) prostatectomy cases, post-surgery serum total PSA levels were >0.2ng/ml indicating biochemical failure and the remaining 13/28 (46.43%) had PSA levels of $\leq$0.2ng/ml. This indicated that over 50% of African American men had biochemical failure in our relative small series of cases. Post-prostatectomy data showed 28.57% (8/28) had developed aggressive prostate cancer, 50% (14/28) had not developed aggressive cancer and aggressiveness was unknown in 21.43% (6/28) of cases. Ratios of CB to SA in biopsy cases were significantly elevated in Gleason score 6 (p=0.02) and score 7 (p=0.004) tumors. In contrast, ratios of CB to SA in BPH and cancer of Gleason score 6 prostatectomy samples were not significant (p=0.30) when compared to BPH, but CB to SA ratios were significant (p=0.05) in Gleason score 7 prostatectomy samples. Our finding of high CB to SA ratios are consistent the idea that these markers can define aggressive prostate cancer. For a definitive conclusion, additional samples are being evaluated.

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Study #2

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Supporting Data:

Study #1

Table 1
Distribution of Prostate Cancer Patients With Gleason Score 6 Tumors

Number of Biopsy Samples	65
Caucasian	54
African American	11
Pre-Prostatectomy Data	
Age at Prostatectomy Mean±SEM (Range)	62.7±0.8 (47-73)
Gleason Score 6 Tumors	65
Presurgery PSA Mean±SEM (Range)	6.7±0.5 (1.25-20)
Clinical Stages	
T1c	1
T2a	14
T2b	13
T2c	34
T3a	2
T3b	1
Post-Prostatectomy Data	
Number of Years since RRP Mean±SEM (Range) *	6.68±0.79
Postsurgery PSA Mean±SEM (Range)	0.02±0.01 (0-0.62)
Number of Patients with PSA<0.2ng/ml	63
PSA±SEM (Range)	0.003±0.02 (0-0.12)
Number of Patients with PSA>0.2ng/ml	2
PSA±SEM (Range)	0.42±0.29 (0.21-0.62)
Lymph Node Negative	59
Unknown Lymph Node Status	6
Positive capsule/margins	2
Negative capsule/margins	63
Distant Metastasis Negative (by bone scan)	36
Distant Metastasis Negative (clinically)	29
TNM	T1-3 N0-x M0-x

* Used December 31, 2005 as the end date

Table 2

Immunostainings of CB, Stefin A, and CB to Stefin A Ratios in Gleason Score 6 Tumors

Protein Localizations	BPH	PIN	Cancer
CB Mean±SEM (range)	3.14±0.13 (1.48-5.43)	3.34±0.23 (1.39-6.40)	3.26±0.12 (1.43-5.81)
SA Mean±SEM (range)	2.70±0.09 (1.09-4.41)	2.39±0.16 (1.03-3.96)	1.02±0.09 (0.12-3.11)
CB/SA Ratio Mean±SEM (range) *	1.21±0.05 (0.62-2.94)	1.65±0.19 (0.47-4.5)	4.89±0.48 (0.85-19.54)

* The overall mean ratios of CB to stefin A were obtained from the ratio of each individual case.

Statistical significance was determined using Student's t test ($P<0.05$). CB to SA ratios were significant when BPH was compared to PIN ($P=0.036$) and cancer ($P<0.0001$).

Table 3

Patients with biochemical recurrence	Patient 1	Patient 2	Patient 3
CB to SA Ratio in cancer	2.66	4.92	11.46
TNM Stage	T2c N0 M0	T2a N0 M0	T2c N0 M0
Margin status	positive	negative	positive
Race	Caucasian	African-American	Caucasian
Additional treatment	ext. beam radiation	ext. beam radiation	lost to follow-up
Current PSA	undetectable	undetectable	lost to follow-up

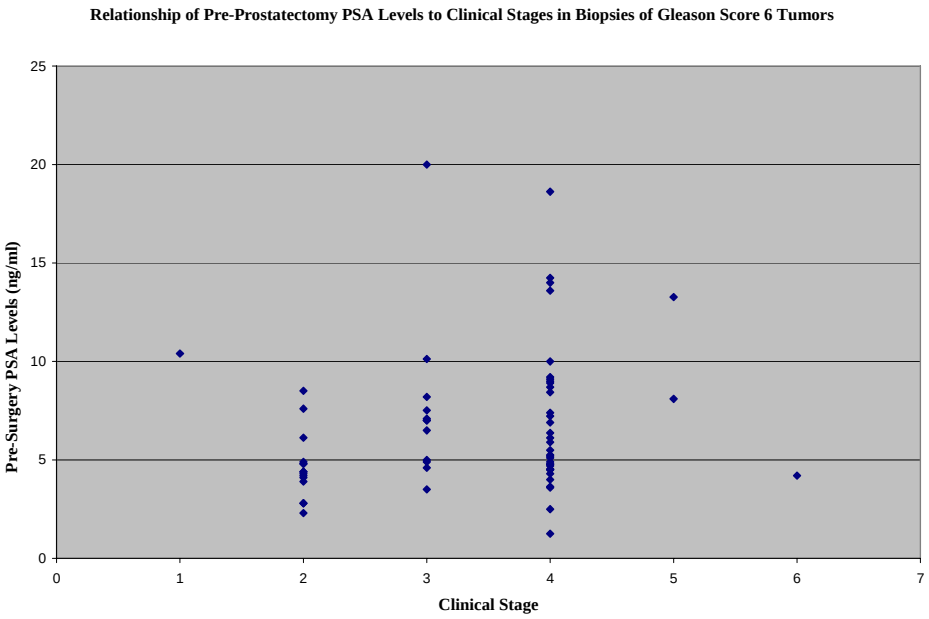


Fig. 1. The figure illustrates 10ng/ml or greater serum total PSA levels in 9/65 (13.8%) cases. The remaining 56/65 (86.2%) cases had lower serum total PSA levels. The majority of biopsy patients had T2a, T2b, and T2c clinical stages.

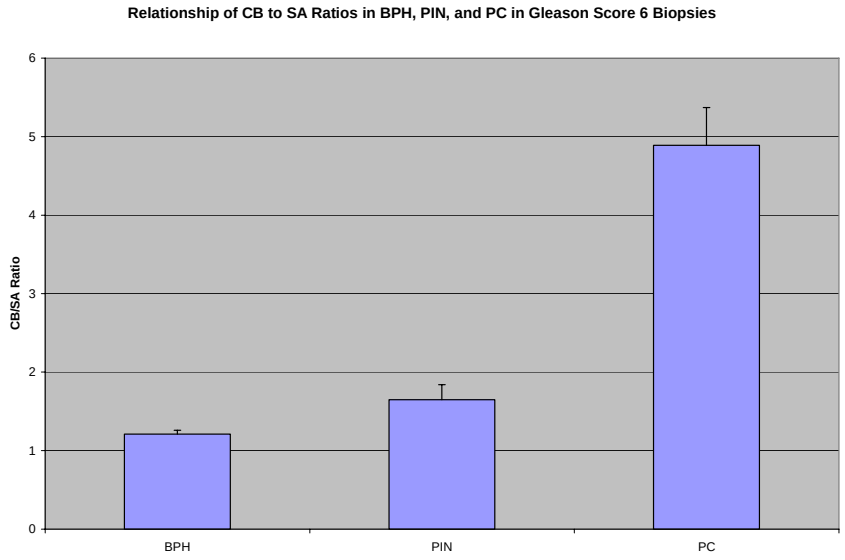


Fig. 2. The bar graph illustrates CB to SA ratios in BPH, PIN and PC. The ratios were significantly higher in PIN ($P=0.036$) and PC ($P<0.0001$) when compared to BPH. PC had significantly higher ratios than PIN ($P<0.0001$). Error bar=SEM.

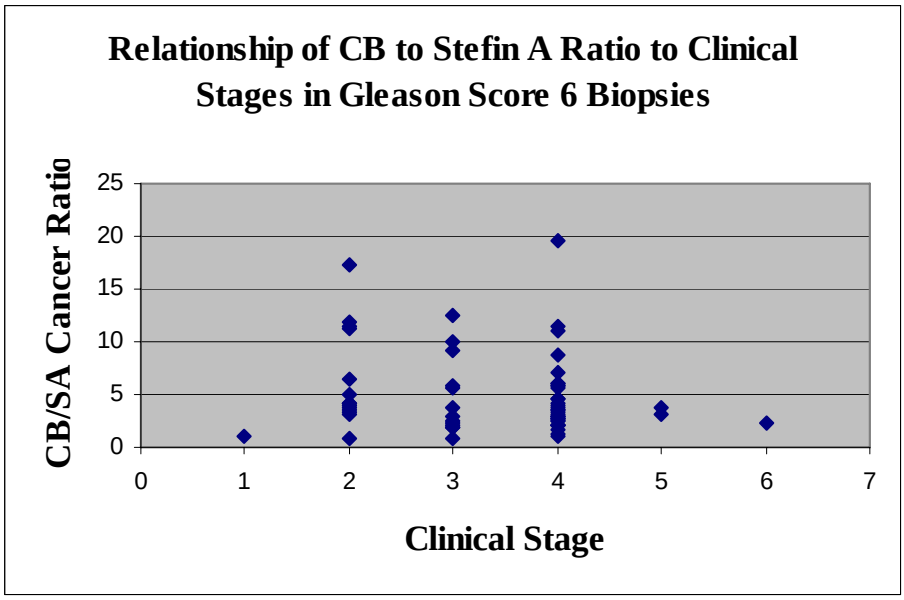


Fig. 3. The figure illustrates CB to SA ratios of 10 and higher in 9/65 (13.8%) cases. The remaining 56/65 (86.2%) cases had lower ratios CB to SA ratios in which 11/65 (16.9%) cases were between 5 and 10. The distribution of ratios was associated with T2a, T2b, and T2c clinical stages.

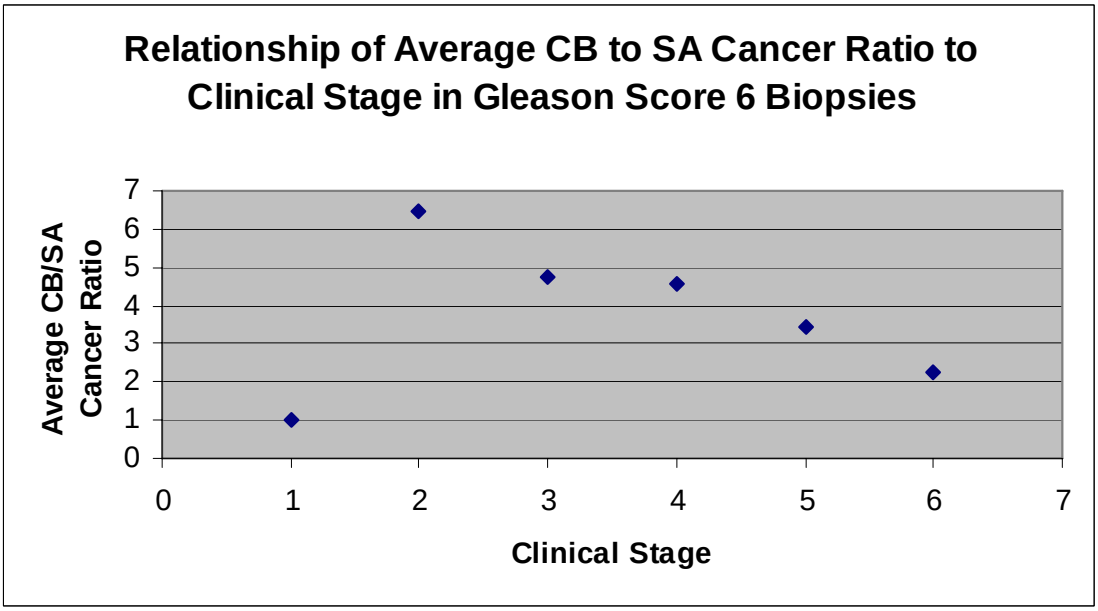


Fig. 4. Figure shows an inverse relation of CB to SA ratios to clinical stages in T2a-T3b except in a single case showing T1c stage.

Figure 5.

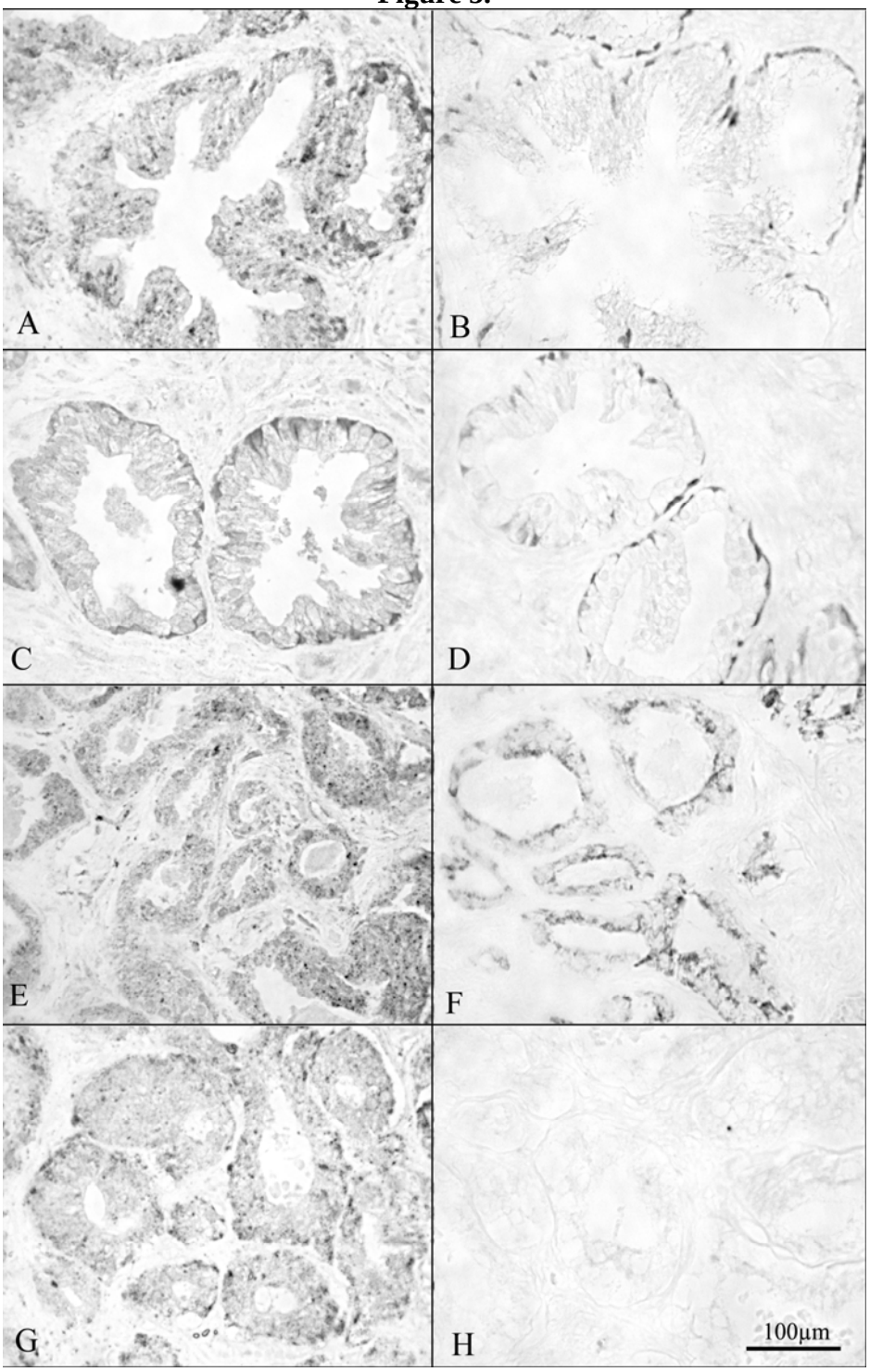


Fig. 5. Immunohistochemical Localization of Cathepsin B and Stefin A in Gleason Score 6 Biopsies

A. Micrograph shows immunohistochemical localization of CB in basal and columnar cells of BPH glands. (Case # VU 74).

B. Micrograph illustrates immunostaining of SA in basal and columnar cells of BPH glands. The ratio of CB to SA was about 1.48.

C. Micrograph illustrates CB immunostaining in basal cells of PIN. (Case # VU 49).

D. Immunostaining of SA in basal and columnar/cuboidal cells of PIN. The ratio of CB to SA was about 1.35.

E. Micrograph illustrates decreased level of CB immunostaining in a Gleason score 6 tumor when compared to CB in figure G. (Case # VU51).

F. Immunostaining for SA in a Gleason score 6 tumor. The ratio of CB to SA was about 0.93.

G. Micrograph illustrates increased CB immunostaining in a Gleason score 6 tumor when compared to CB in figure E. (Case # VU74).

H Immunostaining of SA decreased in columnar/cuboidal cells of Gleason score 6 tumor. The ratio of CB to SA was about 20.5. A bar illustrates magnifications of all figures.

Study #2

Table 1. Distribution of Gleason Histological Scores in Needle Biopsies

Gleason Score	6	7	8	Total
[1]Number of Samples	7	14	3	24
[2]PSA, Mean±SEM	5.95±1.07	8.42±2.09	-	7.46±1.34
Range	1.40 - 9.18	0.92 - 22.50	-	0.92 - 22.50
No PSA data	-	3	3	6 (25.00%)
[3]TNM (Jewett-Whitmore)				
Normal	1	-	-	1 (4.17%)
T2c (B2, B3)	1	3	1	5 (20.83%)
T3a (C1)	1	3	-	4 (16.67%)
Unknown	4	8	2	14 (58.33%)

SEM = Standard Error of the Mean

[1]Twelve cases were not included because of unavailable slides or cancer in sections.

[2]Pre-RP surgery serum total PSA levels.

[3] TNM and Jewett Whitmore classifications are cited from Ellis WJ, and Lange PH. Prostate Cancer. Endocrinol Metab Clin North Am., 1994; 23:809-824.

Table 2. Distribution of Gleason Histological Scores After Radical Prostatectomies of African American Patients

Gleason Score	6	7	8	Total
[1]Number of samples	16	11	1	28
Range of age at surgery	48 - 74	54 - 70	58	48 - 74
Mean±SEM	62.92±2.01	61.62±1.66	-	62.24±1.31
[2]Serum PSA levels > 0.2ng/ml	6	8	1	15 (53.57%)
Serum PSA levels ≤ 0.2ng/ml	10	3	-	13 (46.43%)
TNM (Jewett-Whitmore)				
T2a - T2c (B1, B2, B3)	9	2	-	11 (39.29%)
T3a - T3c (C1, C2)	4	3	-	7 (25.00%)
N1 - N3 (D1)	-	1	1	2 (7.14%)
Unknown	3	5	-	8 (28.57%)
[3]Aggressive PC	1	6	1	8 (28.57%)
Non-Aggressive PC	10	4	-	14 (50.00%)
Unknown status	5	1	-	6 (21.43%)

PC = Prostate Adenocarcinoma

[1]Eight cases were not included because of unavailable slides or cancer in sections.

[2]PSA > 0.2ng/ml indicated biochemical failure.

[3]Aggressive PC was defined by the presence of cancer cells in seminal vesicle and/or pelvic lymph nodes.

Table 3. Immunohistochemical Distribution of Cathepsin B, Stefin A, and Their Ratios in Gleason Score 6-8 Biopsies and Prostatectomies

	# of Cases	CB (Range)	SA (Range)	CB/SA Ratio (Range)
Biopsy				
[1]BPH	15	0.78 ± 0.10 (0.33-1.64)	1.38 ± 0.19 (0.35-2.89)	0.86 ± 0.20 (0.19-3.08)
[2]Gleason 6	3	0.72 ± 0.20 (0.37-1.07)	0.17 ± 0.05 (0.06-0.24)	4.59 ± 0.62 (3.60-5.74)
Gleason 7	13	0.57 ± 0.05 (0.26-0.91)	0.28 ± 0.05 (0.08-0.62)	3.53 ± 0.81 (0.62-10.41)
Gleason 8	3	0.65 ± 0.10 (0.48-0.83)	0.55 ± 0.24 (0.19-1.01)	1.75 ± 0.83 (0.81-3.40)
Prostatectomy				
[3]BPH	23	0.43 ± 0.06 (0.11-1.12)	0.91 ± 0.17 (0.11-2.57)	0.92 ± 0.17 (0.10-3.28)
[4]Gleason 6	10	0.26 ± 0.04 (0.06-0.52)	0.17 ± 0.03 (0.04-0.37)	1.56 ± 0.16 (0.91-2.56)
[5]Gleason 7	9	0.28± 0.06 (0.10-0.62)	0.29 ± 0.08 (0.05-0.65)	1.70 ± 0.50 (0.35-4.91)
Gleason 8	1	0.13	0.25	0.51

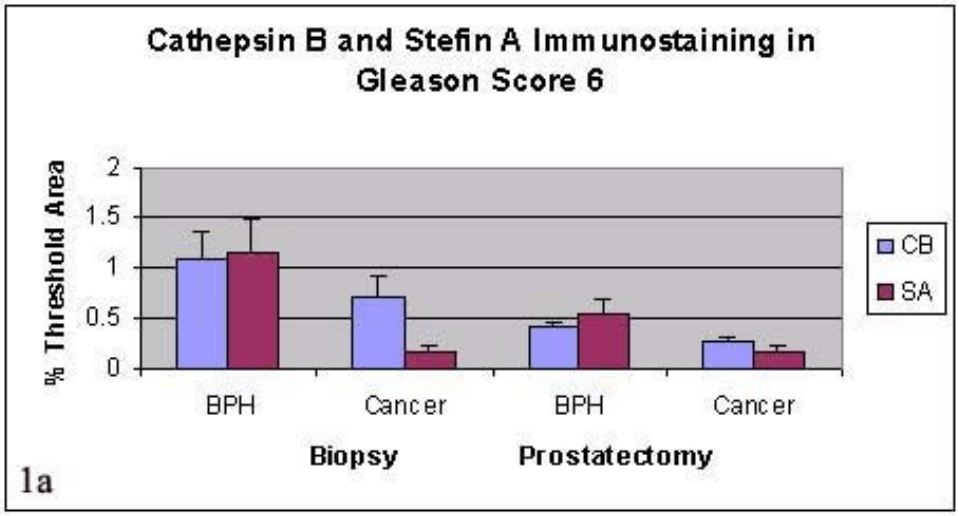
[1]Two BPH biopsy cases were considered outliers due to high CB immunostaining or high CB to stefin A ratio. The outliers did not follow patterns of immunostaining found in other cases.
[2]Three Gleason score 6 biopsy cases were considered outliers due to high CB or stefin A immunostaining, or high CB to stefin A ratio.
[3]One BPH prostatectomy case was considered an outlier due to high CB to stefin A ratio.
[4]Six Gleason score 6 prostatectomy cases were considered outliers due to high CB immunostaining, stefin A immunostaining and/or CB to stefin A ratio.
[5]Two Gleason score 7 prostatectomy cases were considered outliers due to high CB or stefin A immunostaining.

Table 4. Distribution of Cathepsin B to Stefin A Ratios in Aggressive PC Biopsies

PC Patients	Biopsy - CB/SA Ratio # of patients (mean ± SEM)	Prostatectomy - CB/SA Ratio # of patients (mean ± SEM)
[1]Aggressive	6 (1.79 ± 0.58)	7 (1.64 ± 0.58)
Non-Aggressive	10 (4.52 ± 0.91)	10 (1.56 ± 0.27)
Unknown	8 *	11 **

* Includes four outliers and four unknown aggressiveness
** Includes five outliers and six unknown aggressiveness

[1]Aggressive PC was defined by cancer cell positive seminal vesicles and/or pelvic lymph nodes.



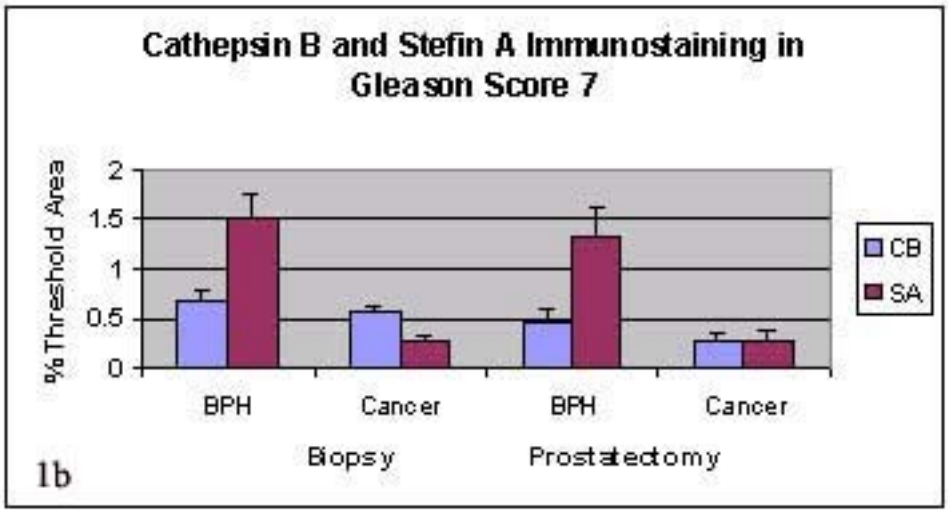


Figure 1a shows that CB alone and SA alone were similar in biopsy and prostatectomy sections of BPH. Stefin A was lower in biopsy and prostatectomy sections when compared to CB in Gleason score 6 cancer.

Figure 1b shows that SA alone is higher in comparison to CB in biopsy and prostatectomy sections of BPH. Stefin A alone was lower in cancer when compared to CB alone in Gleason score 7 biopsies. However, CB alone and SA alone were similar in prostatectomy samples of Gleason score 7 tumors.

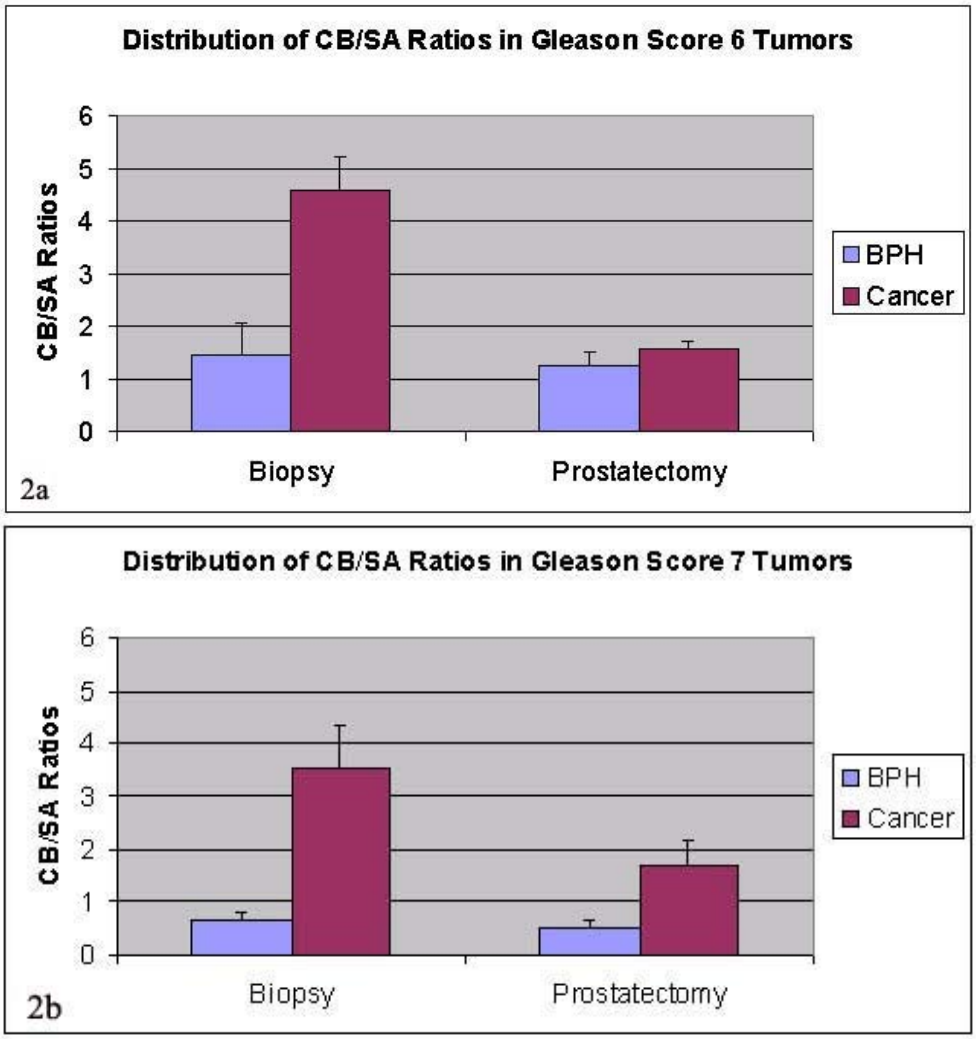


Figure 2a and b show CB to SA ratios in cancer that were significantly elevated in Gleason score 6 (p=0.02) and Gleason score 7 (p=0.004) biopsy samples when compared to BPH. CB to SA ratios in BPH and cancer of Gleason score 6 prostatectomy sections were not significant (p=0.30), however the ratios were significant in Gleason score 7 prostatectomies (p=0.05). CB to SA ratios in biopsy were significantly higher than prostatectomy samples in Gleason score 6 cancer (p=0.04). The same ratios were not significant in Gleason score 7 cancer (p=0.07).

Figure 3

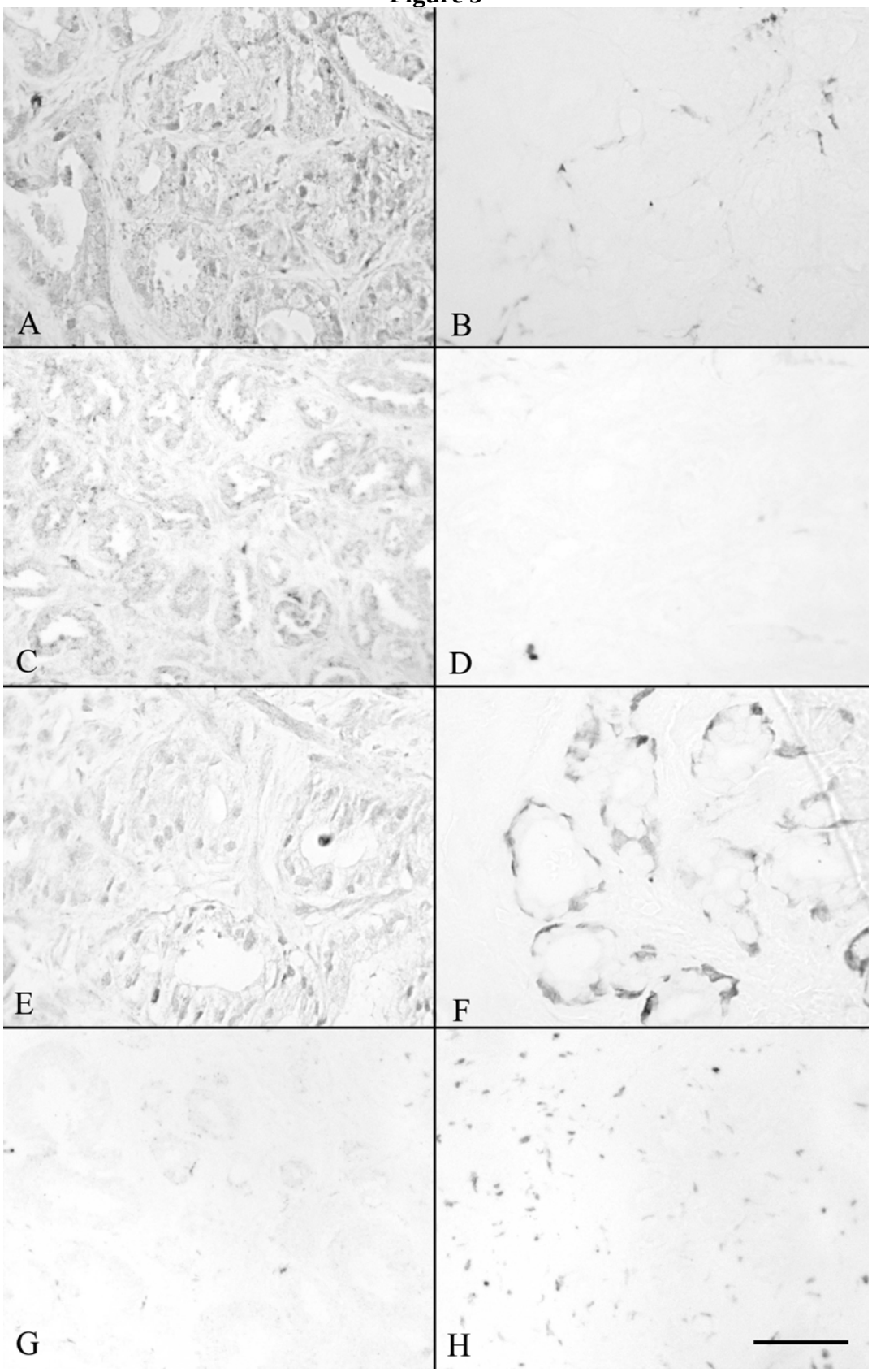


Figure 3. Immunohistochemical Localization of Cathepsin B and Stefin A in African American Biopsies and Prostatectomies

A. Micrograph shows elevated CB immunostaining in cuboidal/columnar and isolated cancer cells in a Gleason score 6 biopsy.

B. Micrograph illustrates decreased SA in a Gleason score 6 biopsy from the same patient in figure 3A. The CB to SA ratio was 3.87.

C. Micrograph illustrates a Gleason score 6 prostatectomy with elevated CB immunostaining.

D. Immunostaining for SA is low in a Gleason score 6 prostatectomy from the same patient in figure 3C. The CB to SA ratio was 10.39.

E. Immunostaining of CB in cancer cells in a Gleason score 7 biopsy of an African American. The CB levels are lower in comparison to figure 3A.

F. Micrograph illustrates a Gleason score 7 biopsy with SA immunostaining from the same patient in figure 3E. The levels are elevated when compared to figure 3B. The CB to SA ratio was 0.19.

G. Immunostaining for CB in a Gleason score 7 prostatectomy decreased when compared to figure 3C.

H. Micrograph shows a Gleason score 7 prostatectomy, from the same patient in figure 3G, with elevated SA immunostaining when compared to figure 3D. The CB to SA ratio was 0.21. Bar illustrates magnification of images. It is equal to 50μm in prostatectomies and 100μm in biopsies.

Appendices:

Copies of two manuscripts are attached at the end of the report.

Prognostic Value of the Cathepsin B to Stefin A Ratio in Prostate Needle Biopsies

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Running Title: Characterization of Prostate Cancer by Cathepsin B and Stefin A

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Abstract

We have previously demonstrated a significant positive association between pelvic lymph node metastases and increased expression of cathepsin B (CB) relative to its inhibitor stefin A (SA) in patients with prostate cancer (PC) who underwent radical prostatectomy (RP). Our objective in this study was to evaluate these markers in prostate needle biopsies showing Gleason histological score 6 tumors. Formalin-fixed, paraffin embedded specimens from 65 patients were subjected to immunohistochemical (IHC) localization of CB (mouse anti-human CB IgG) and SA (mouse or goat anti-human SA IgG). Immunostaining was quantified using a computer-based image analysis system equipped with Metamorph software. Data were analyzed using univariate and multivariate techniques, and with Student's t-test ($p<0.05$) for statistical significance. At initial diagnosis, the age of patients ranged from 47 to 73 years and all had Gleason histological score 6 tumors both in needle biopsies and RP specimens, with pre-surgery PSA values ranging from 1.25 to 20.0 ng/ml (6.7 ± 0.5: mean ± standard error of the mean, SEM). The clinical stages of PC ranged from T1c to T3b. Reaction products for CB and SA were found in the cytoplasm of neoplastic cells, and basal and some columnar/cuboidal cells of benign prostatic hyperplasia (BPH) glands and prostatic intraepithelial neoplasia (PIN) glands. Ratios of CB to SA ranged from 0.62 to 2.94 with a mean of 1.21 ± 0.05 in BPH, 0.47 to 4.5 with a mean of 1.65 ± 0.19 in PIN, and 0.85 to 19.54 with a mean of 4.89 ± 0.48 in PC. CB immunostaining alone was not statistically different in PIN and cancer, but SA alone was significantly lower in PC ($P< 0.0001$) than in BPH and PIN glands. Ratios of CB to SA were significantly higher in cancer and PIN when compared to BPH ($P<0.0001$). Ratios of CB to SA in turn were significantly higher in cancer than in PIN ($P<0.0001$). Our data show that PC is a heterogenous disease within a single Gleason grade with respect to CB to SA ratios. No correlation was found between high CB to SA ratios and adverse outcome for Gleason score 6 tumors since the number of such patients was low and the follow-up period was short for most of the patients. Long-term follow-up may help stratify PC into aggressive and less aggressive clones based on CB to SA ratios and assist in selection of optimum treatment.

Introduction

Prediction of invasiveness and progression of prostate cancer (PC), a complex and heterogeneous disease, is a critical factor in selecting specific treatments such as radical prostatectomy (RP), brachytherapy/external beam radiation, chemotherapy, hormone therapy, immunotherapy, and/or watchful waiting (1-4). Presently, treatment decisions are based on relatively few pre-treatment prognostic factors such as needle biopsy Gleason grade, serum total prostate specific antigen (PSA), percent free PSA, and clinical stage (5-10). Additional parameters such as DNA ploidy, proliferation markers, microvessel density, nuclear morphometry and others have been assessed for predicting invasive potential of PC, but they have been of limited value (11). Within a single Gleason score/grade, some patients have aggressive disease and die within 5 years while others with less aggressive disease survive 10 years or longer (9, 12-14). Therefore, selection of an appropriate treatment is critical for survival and an acceptable quality of life.

We hypothesized that patients opting for a treatment based on limited prognostic factors could benefit from assessment of their biopsy samples by several molecular biomarkers that have been shown to be involved in cancer cell invasion and progression to other organs. We have previously demonstrated that the increased ratio of cathepsin B (CB) – an enzyme involved in degradation of basement membrane (BM) and extracellular matrix (ECM) – to its inhibitor stefin A (SA) shows a significant correlation with pelvic lymph node metastases in patients with PC who have undergone RP (15). Our objective in this retrospective study was to evaluate the prognostic value of these markers in prostate needle biopsy samples by immunohistochemical (IHC) methods.

Materials and Methods

We obtained 65 cases of PC with Gleason score 6 tumors in both needle biopsies and RP specimens from the archives of Virginia Urology Center (Richmond, VA) after obtaining approval from the Institutional Review Board. Since Gleason grade is one of the most powerful independent prognostic factors in PC, it was decided to study cases within a single Gleason grade to minimize the influence of other factors on the outcome (Table 1). All tissue sections were graded according to the Gleason grading system (6,7) by one of us (DMR). Data on surgery date, pre-and post-RP PSA, clinical stage, tumor volume, margin/capsule status, lymph node status, and metastasis were collected. Sections from formalin-fixed, paraffin-embedded needle biopsy blocks were cut at 5 μm and subjected to immunostaining with antibodies to CB and SA.

Immunohistochemical Localization of Cathepsin B and Stefin A: Mouse anti-human CB IgG (IM27L) was obtained from Oncogene Research Products (Calbiochem, Cambridge, MA). Mouse monoclonal anti-human SA IgG was purchased from KRKA (Novo Mesto, Slovania) and goat anti-human cystatin (stefin) A IgG from R& D Systems (Minneapolis, MN). Antibodies were affinity purified using immobilized protein A or human SA by the manufacturer. Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). We localized CB and SA in biopsy sections using IHC techniques reported by us (16, 17). Briefly, antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0) using Decloaking Chamber Pro machine (Biocare Medical, Walnut Creek, CA). Mouse anti-CB antibody IgG localized CB and mouse or goat anti-human SA IgG localized SA in adjacent sections. Since the number of biopsy sections was limited, prostatectomy sections were used for negative control. These controls were incubated with pre-immune mouse or goat serum in lieu of the primary antibodies. The reaction products were developed, usually less than 10 min, with fresh-filtered 3, 3'-diaminobenzidine (DAB) solution (0.25 mg/ml; Sigma) in phosphate buffer saline (PBS) with 0.01% H_2O_2 as the substrate. Chromogenic development was viewed through a light microscope and reaction product was enhanced with osmium tetroxide solution.

Quantification of Localization Data by Metamorph Image Analysis System: Immunostaining for CB and SA were quantified using a computer-based image analysis system equipped with Metamorph software (Universal Imaging Corp., West Chester, PA), as reported by us (18-20). Briefly, images of reaction products for CB and SA were acquired at a magnification of 400 X directly from the microscope slides to a computer using a digital camera (Photometrics, Tucson, AZ) attached to a Zeiss microscope. On the basis

of gray values ranging from 4095 to 0, white to black, respectively, threshold boundaries of immunostaining were created. All immunostained objects included within the designated gray value range were expressed as a percentage of the total field area under view at the selected magnification. Data are presented as mean ± standard error of the mean (SEM). Data were analyzed using univariate and multivariate techniques. Statistical significance was determined using Student's t-test ($p<0.05$).

Results

Profile of Prostate Cancer Patients: The age of PC patients at the initial diagnosis ranged from 47 to 73 years (mean ± standard error of the mean (SEM) of 62.7 ± 0.8) (Table 1). All patients had Gleason score 6 tumors both in prostate needle biopsies as well as RP specimens. The regional pelvic lymph nodes were negative for cancer cells in 59 patients (59/65, 90.8%) and node status was unknown in six cases (6/65, 9.2%). None of the patients had evidence of distant metastases based on bone scan (36/65 cases, 55.4%) and/or clinical data (29/65 cases/44.6%). The clinical stages ranged from T1c to T3b with the majority of patients showing stage T2c tumors (34/65, 52.3%), T2a (14/65, 21.5%), and T2b (13/65, 20%) (Table 1). The number of cases with T1c, T3a and T3b stages was limited. Pre-surgery PSA ranged from 1.25 to 20.0 ng/ml (mean of 6.7 ± 0.5) (Table 1, Fig.1). Pre-surgery PSA levels of less than 9 ng/ml were associated with stage T2a, while T2b and T2c stages showed considerable variations in PSA levels. In our series, 9/65 (13.8%) cases had 10 ng/ml and higher serum total PSA levels (Fig. 1). Fifty-five (55/65, 84.6%) patients had pre-RP serum PSA levels less than 10ng/ml and the remaining patient had unknown levels. The post-surgery PSA levels were taken between 2 months and 7.5 years after the RP (mean 4.9 ± 1.35 years) and ranged from 0.0 to 0.62 ng/ml (mean 0.02 ± 0.01). Three patients with pre-surgery PSA levels of 2.8, 5.9, and 3.64 ng/ml, showed evidence of biochemical recurrence of PC after 4.35, 5.03, and 4.00 years, respectively, in post-surgery clinical data (Table 3). One patient had a post-surgery PSA level above 0.1 ng/ml, while the other two patients had post-surgery PSA levels above 0.2 ng/ml. PSA levels had not increased in the remaining 62 patients.

Immunostaining of Cathepsin B and Stefin A in Benign Prostatic Hyperplasia Glands: CB and SA immunostaining was present predominantly in the cytoplasm of basal cells and some cuboidal/columnar cells of BPH glands (Fig. 5 A, B). Immunostaining of CB ranged from 1.48 to 5.43, with a mean of 3.14 ±

0.13 (Table 2). Likewise, immunostaining of SA ranged from 1.09 to 4.41, with a mean of 2.70 ± 0.09. The ratios of CB to SA ranged from 0.62 to 2.94, with a mean of 1.21 ± 0.05 (Table 2).

Immunostaining of Cathepsin B and Stefin A in PIN Glands: In PIN glands, the two markers localized in the basal cells (Fig.5 C, D). Immunostaining of CB ranged from 1.39 to 6.40, with a mean of 3.34 ± 0.23. Likewise, SA localization ranged from 1.03 to 3.96, with a mean of 2.39 ± 0.16. The ratios of CB to SA ranged from 0.47 to 4.5, with a mean of 1.65 ± 0.19 (Table 2).

Immunostaining of Cathepsin B and Stefin A in Prostate Cancer: CB and SA localized to the neoplastic cells in the malignant glands (Fig.5 E-H). The distribution of CB and SA protein reaction products showed considerable variations within Gleason score 6 tumors that are considered histologically and morphologically similar. We have previously noted this phenomenon in RP specimens (15). Immunostaining of CB ranged from 1.43 to 5.81, with a mean of 3.26 ± 0.12. SA localization ranged from 0.12 to 3.11, with a mean of 1.02 ± 0.09. The ratios of CB to SA ranged from 0.85 to 19.54, with a mean of 4.89 ± 0.48 (Table 2) (Fig. 5 E-H).

Analysis of Cathepsin B and Stefin A Immunostaining Data: The immunostaining pattern of BPH glands was used as a control for data analysis. Differences in immunostaining of CB alone were not statistically significant in BPH, PIN and cancer, but SA alone was significantly lower in PC ($P < 0.0001$) when compared to BPH glands and PIN (Table 2). Ratios of CB to SA were significantly higher in cancer when compared to BPH glands and PIN ($P<0.0001$) and also in BPH glands compared to PIN ($P=0.036$) (Table 2) (Fig. 2). Three patients with rising PSA levels (biochemical recurrence) had a clinical stage of T2c, T2c, and T2a and CB to SA ratios of 2.66, 4.92, and 11.46 in cancer areas, respectively (Table 3). In contrast, CB to SA ratios in BPH glands of two of the cases were 1.42 and 1.16 respectively. Thus, ratios of CB to SA were significantly lower in the BPH glands of these two cases when compared to malignant foci. The CB to SA ratio in the BPH glands in the third case with biochemical recurrence could not be computed due to paucity of BPH glands in the specimen.

Cathepsin B and Stefin A Ratios in Relation to Clinical Stages and PSA Levels: Our data showed that higher CB to SA ratios were predominantly associated with T2a, T2b and T2c clinical stages, and a few cases had T1c, T3a and T3b stages (Fig. 3). The average ratios of CB to SA showed an inverse relationship to T2a to

T3b clinical stages (Fig. 4). Likewise, patients with T2a, T2b, and T2c stages were associated with CB to SA ratios that ranged from 0.85 to 19.54, with a mean of 5.04 ± 0.50. The single case of T1c did not show the above pattern. Patients with T2a, T2b and T2c clinical stages were associated with variable pre-RP serum PSA levels that ranged from 1.25 to 20, with a mean of 6.56 ± 0.47 (Fig. 1). Nine patients (9/65, 13.8%) had PSA levels ≥ 10 ng/ml and these were associated with T2b, T2c, and T3a clinical stages (Figs. 1, 4). Fifty-five (55/65, 84.6%) patients had pre-RP serum PSA levels less than 10ng/ml and the remaining patient had unknown levels.

Discussion

Prostate cancer is a heterogenous disease and this phenomenon was well recognized by Gleason in his grading system (6, 7). In one study of RP specimens, an average of 2.7 Gleason grade patterns (range 1-5) were found (21). However, this heterogeneity is not accounted for by morphologic or histologic criteria within a single Gleason grade in a given specimen. Within a single Gleason score/grade, some patients have aggressive disease and die within 5 years while others with less aggressive disease survive 10 years or longer (9, 12-14). The prognostic factors currently used in PC do not always accurately predict tumor aggressiveness. The need for a reliable prognostic marker is greatest in situations where one encounters a limited focus of cancer in one or two of the cores in an extended prostate biopsy protocol (8 or more cores). An ideal marker would help predict clinically insignificant organ-confined cancer with little risk of extra-prostatic spread. We have previously demonstrated that the ratio of CB to SA has the potential of identifying subpopulations of aggressive and less aggressive PCs within a single Gleason score in RP specimens (15). We found three distinct staining patterns: CB greater than SA, CB less than SA, and CB equal to SA. Our data showed a significant association between high ratios of CB to SA (CB > SA) and pelvic lymph node metastases.

In the current study, we studied the predictive value of CB to SA ratios in prostate needle biopsies. The cases were selected within a single Gleason score 6 to avoid the influence of Gleason grade, one of the most powerful predictors of outcome in patients with PC. Cathepsin B, a cysteine protease, is involved in the degradation of BM and ECM proteins and is associated with progression in PC and other solid tumors (18-20, 22, 23). Since it is regulated by its endogenous inhibitor, stefin A, ratios of CB to SA provide better prediction of human PC progression than CB or SA alone in biological compartments. Our analysis of prostate biopsies

indicated significantly higher ($P<0.0001$) ratios of CB to SA in malignant glands when compared to BPH glands and PIN areas in the same case. In the entire group, considerable heterogeneity was found in the expression ratios of CB to SA (mean 4.89 ± 0.48; range 0.85 to 19.54). This is similar to the pattern previously found by us in RP specimens (15). Three patients in our series had biochemical failure shown by serum PSA levels rising to 0.1 ng/ml or higher (Table 3). Two of these three cases had positive resection margins in RP specimens. Two of the three cases were given external beam radiation and had undetectable PSA at last follow-up. The third patient moved and was lost to further follow-up. The CB to SA ratios in these cases were 2.66, 4.92, and 11.46. Due to the small number of adverse events in our series, statistical correlation between elevated CB to SA ratio and the risk of biochemical recurrence was not attempted. Nine of 65 (13.8%) cases showed CB to SA ratio greater than 10. Only one of these 9 showed biochemical recurrence (mentioned above); the remaining 8 cases showed no evidence of disease at last follow-up.

We acknowledge several limitations in this study that may explain the failure to find correlation between CB to SA ratios and patient outcome. The number of cases in this series is quite small and only 3 of 65 (4.6%) patients showed evidence of biochemical recurrence. The initial sample size was 100 cases; however, the foci of cancer were exhausted in many of the paraffin blocks, decreasing the number of cases. This limitation could seriously hamper the widespread application of this marker even if it were found to be useful. The mean follow-up period in our study was 6.68 years. Many of the cases in our study with elevated CB to SA ratio have been followed for less than 5 years. Long-term follow-up of the entire group is planned to see if any patterns emerge.

Similar studies with larger sample size and longer follow-up are needed to further elucidate the utility of these markers in prostate needle biopsies. If found useful in stratifying PC patients into potentially aggressive and less aggressive categories, they could assist in selection of appropriate initial therapy. Patients with less aggressive tumors could be offered watchful waiting whereas those with tumors more likely to progress could be treated more aggressively. They could also help select candidates who might benefit from adjuvant therapy following RP.

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Table 1
Distribution of Prostate Cancer Patients With Gleason Score 6 Tumors

Number of Biopsy Samples	65
Caucasian	54
African American	11
Pre-Prostatectomy Data	
Age at Prostatectomy Mean±SEM (Range)	62.7±0.8 (47-73)
Gleason Score 6 Tumors	65
Presurgery PSA Mean±SEM (Range)	6.7±0.5 (1.25-20)
Clinical Stages	
T1c	1
T2a	14
T2b	13
T2c	34
T3a	2
T3b	1
Post-Prostatectomy Data	
Number of Years since RRP Mean±SEM (Range) *	6.68±0.79
Postsurgery PSA Mean±SEM (Range)	0.02±0.01 (0-0.62)
Number of Patients with PSA<0.2ng/ml	63
PSA±SEM (Range)	0.003±0.02 (0-0.12)
Number of Patients with PSA>0.2ng/ml	2
PSA±SEM (Range)	0.42±0.29 (0.21-0.62)
Lymph Node Negative	59
Unknown Lymph Node Status	6
Positive capsule/margins	2
Negative capsule/margins	63
Distant Metastasis Negative (by bone scan)	36
Distant Metastasis Negative (clinically)	29
TNM	T1-3 N0-x M0-x

* Used December 31, 2005 as the end date

Table 2

Immunostainings of CB, Stefin A, and CB to Stefin A Ratios in Gleason Score 6 Tumors

Protein Localizations	BPH	PIN	Cancer
CB Mean±SEM (range)	3.14±0.13 (1.48-5.43)	3.34±0.23 (1.39-6.40)	3.26±0.12 (1.43-5.81)
SA Mean±SEM (range)	2.70±0.09 (1.09-4.41)	2.39±0.16 (1.03-3.96)	1.02±0.09 (0.12-3.11)
CB/SA Ratio Mean±SEM (range) *	1.21±0.05 (0.62-2.94)	1.65±0.19 (0.47-4.5)	4.89±0.48 (0.85-19.54)

* The overall mean ratios of CB to stefin A were obtained from the ratio of each individual case.

Statistical significance was determined using Student's t test ($P<0.05$). CB to SA ratios were significant when BPH was compared to PIN ($P=0.036$) and cancer ($P<0.0001$).

Table 3

Patients with biochemical recurrence	Patient 1	Patient 2	Patient 3
CB to SA Ratio in cancer	2.66	4.92	11.46
TNM Stage	T2c N0 M0	T2a N0 M0	T2c N0 M0
Margin status	positive	negative	positive
Race	Caucasian	African-American	Caucasian
Additional treatment	ext. beam radiation	ext. beam radiation	lost to follow-up
Current PSA	undetectable	undetectable	lost to follow-up

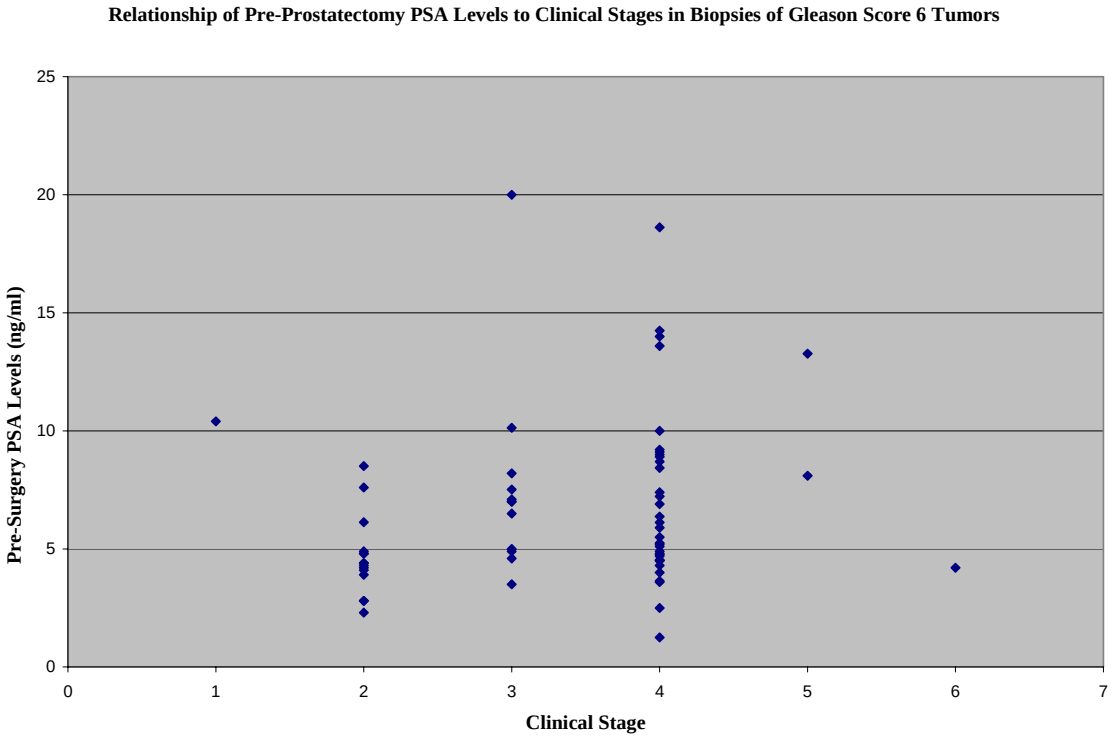

Fig. 1. The figure illustrates 10ng/ml or greater serum total PSA levels in 9/65 (13.8%) cases. The remaining 56/65 (86.2%) cases had lower serum total PSA levels. The majority of biopsy patients had T2a, T2b, and T2c clinical stages.

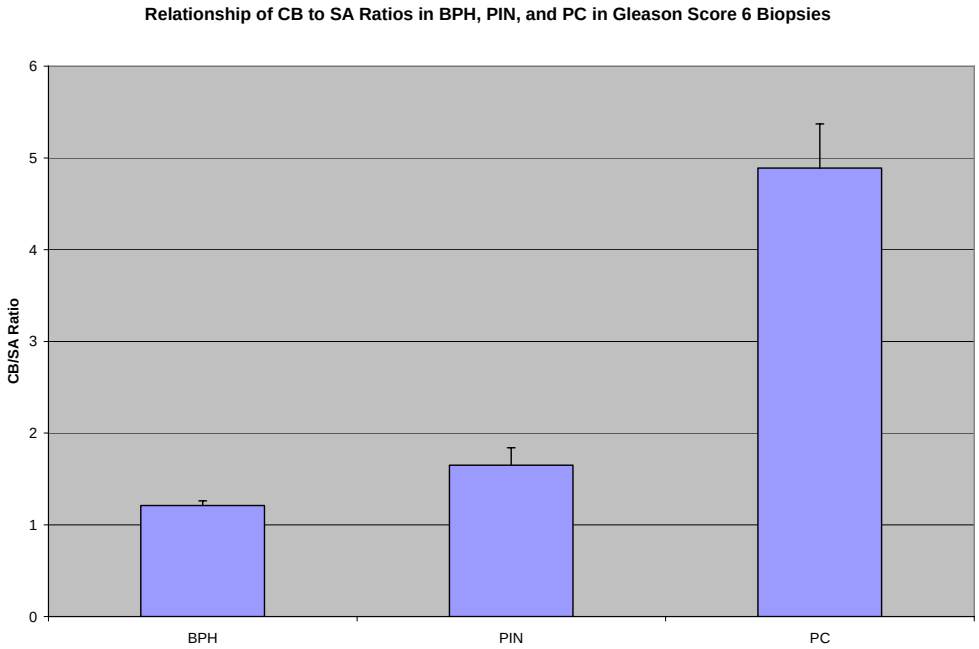

Fig. 2. The bar graph illustrates CB to SA ratios in BPH, PIN and PC. The ratios were significantly higher in PIN ($P=0.036$) and PC ($P<0.0001$) when compared to BPH. PC had significantly higher ratios than PIN ($P<0.0001$). Error bar=SEM.

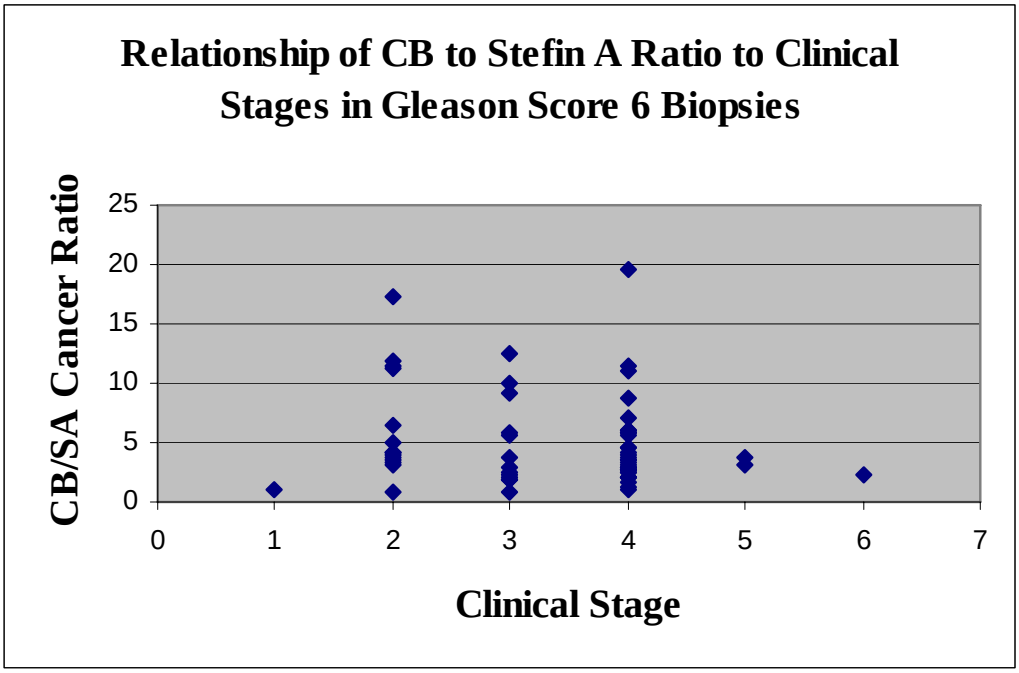

Fig. 3. The figure illustrates CB to SA ratios of 10 and higher in 9/65 (13.8%) cases. The remaining 56/65 (86.2%) cases had lower ratios CB to SA ratios in which 11/65 (16.9%) cases were between 5 and 10. The distribution of ratios was associated with T2a, T2b, and T2c clinical stages.

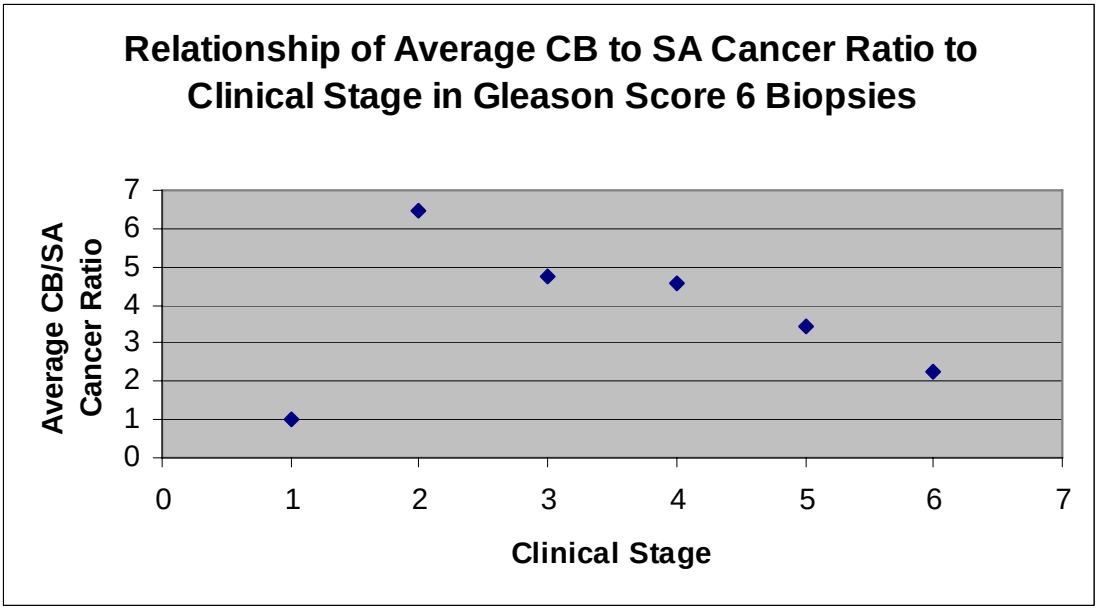

Fig. 4. Figure shows an inverse relation of CB to SA ratios to clinical stages in T2a-T3b except in a single case showing T1c stage.

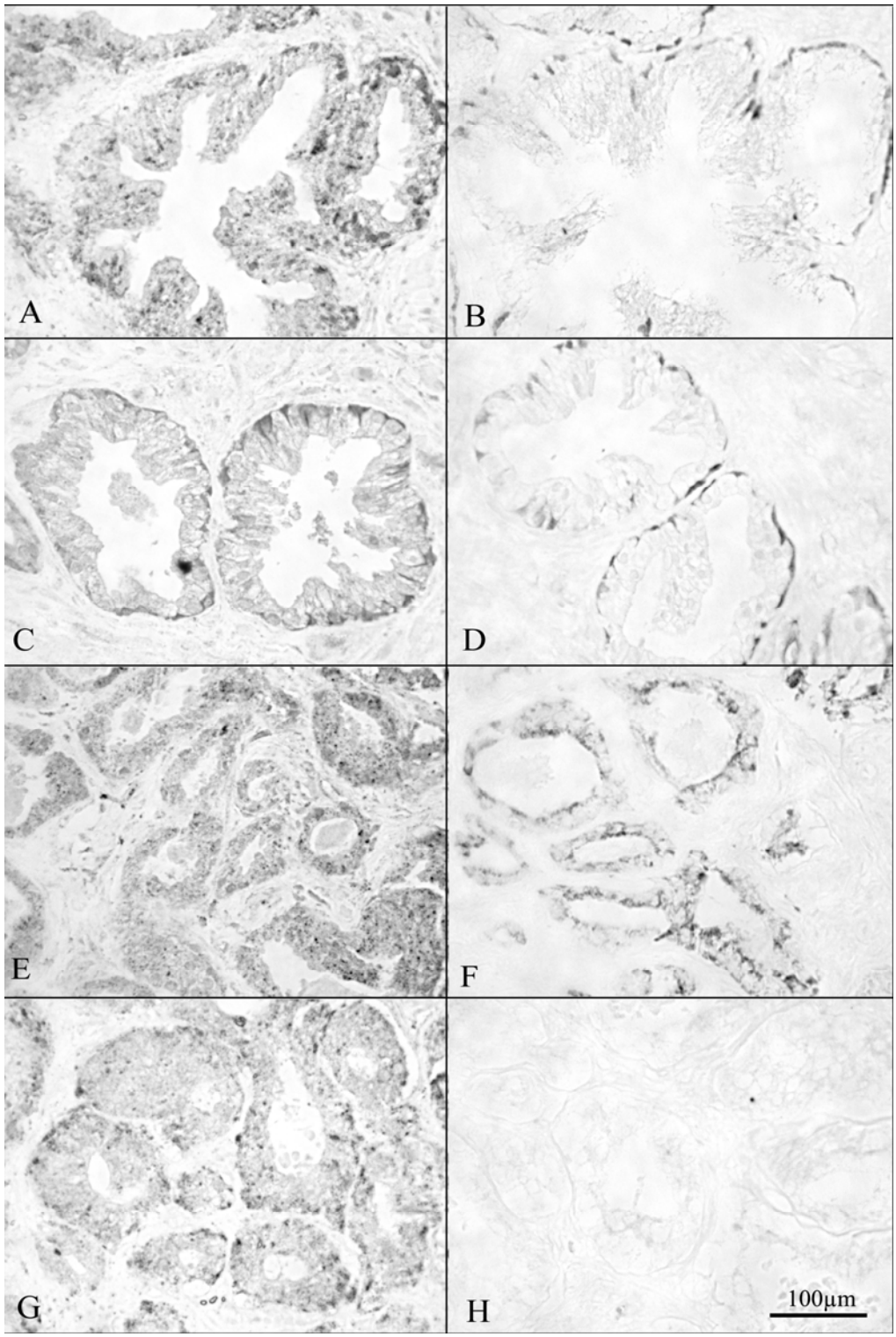

Figure 5

Fig. 5. Immunohistochemical Localization of Cathepsin B and Stefin A in Gleason Score 6 Biopsies

A. Micrograph shows immunohistochemical localization of CB in basal and columnar cells of BPH glands. (Case # VU 74).

B. Micrograph illustrates immunostaining of SA in basal and columnar cells of BPH glands. The ratio of CB to SA was about 1.48.

C. Micrograph illustrates CB immunostaining in basal cells of PIN. (Case # VU 49).

D. Immunostaining of SA in basal and columnar/cuboidal cells of PIN. The ratio of CB to SA was about 1.35.

E. Micrograph illustrates decreased level of CB immunostaining in a Gleason score 6 tumor when compared to CB in figure G. (Case # VU51).

F. Immunostaining for SA in a Gleason score 6 tumor. The ratio of CB to SA was about 0.93.

G. Micrograph illustrates increased CB immunostaining in a Gleason score 6 tumor when compared to CB in figure E. (Case # VU74).

H Immunostaining of SA decreased in columnar/cuboidal cells of Gleason score 6 tumor. The ratio of CB to SA was about 20.5. A bar illustrates magnifications of all figures.

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Characterization of Prostate Cancer in African American Men by Cathepsin B and Stefin A

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Running Title: Analysis of Biopsy and Prostatectomy Samples in the Same Patients

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Abstract

Background: In the era of serum total prostate specific antigen (PSA) measurements, many patients are diagnosed with prostate adenocarcinoma (PC) due to a few cancerous glands and invasive cells in 1 or 2 needle biopsy cores out of 6 to 12 cores. Because PC is complex and heterogeneous, treatment selection based on limited pre-treatment clinical data is often inaccurate. The inability to select aggressive cancers within each Gleason grade greatly affects survival and quality of life of patients. Our objective was to assess the distribution of molecular markers, cathepsin B (CB) and stefin (cystatin) A (SA), in low and high volume tumors in the same patients.

Methods: We evaluated formalin-fixed, paraffin-embedded prostate biopsy and radical prostatectomy (RP) tissue samples from 28 patients by localizing CB (mouse anti-human CB IgG) and SA (goat anti-human SA IgG) by immunohistochemical (IHC) methods. Immunostaining in prostate epithelial cells of cancerous and benign prostatic hyperplasia (BPH) areas was quantified using a computer-based image analysis system equipped with Metamorph software. Data were analyzed using Student's t-test ($p<0.05$) for statistical significance.

Results: The distribution of Gleason histologic scores ranged from 6 to 8 in biopsy and prostatectomy tissue sections. In biopsy patients, pre-surgery serum PSA levels ranged from 0.92 to 22.50 (mean 7.46±1.34 SEM: standard error of the mean) with normal, T2c, T3a, and unknown clinical stages. Post-RP surgery serum PSA levels were >0.2ng/ml in 15 (53.57%) patients and $\leq$ 0.2 ng/ml in 13 (46.43%). The distribution of CB and SA reaction products showed variations between and within Gleason score tumors in both biopsy and prostatectomy tissue sections. Since BPH is not an invasive tumor, it was used as a control for comparing with malignant tumor. Ratios of CB to SA in biopsy cases were significantly elevated in Gleason score 6 ($p=0.02$) and score 7 ($p=0.004$) tumors. In contrast, ratios of CB to SA in BPH and cancer of Gleason score 6 prostatectomy samples were not significant ($p=0.30$), but ratios of CB to SA were significant ($p=0.05$) in Gleason score 7 prostatectomy samples.

Conclusions: Our interim analysis showed that biopsy and prostatectomy sample sizes showing several Gleason scores were too small for any definitive conclusion without addition of new data from additional cases. We are expanding our sample size of biopsy and prostatectomy cases. A definitive conclusion for CB to SA ratios is also premature in this interim report.

Introduction

In the era of serum total prostate specific antigen (PSA) measurements, many patients are diagnosed with prostate adenocarcinoma (PC) due to a few cancerous glands and invasive cells that are found in 1 or 2 needle biopsy cores out of 6 to 12 cores [1-3]. Low volume, 10% PC was found in some patients but others had high volume, 50% tumor, in 1 or 2 biopsy cores [1-4]. Several measurements of pre-treatment PSA levels, pathological grading, clinical stages, and biopsy core tumor volumes are utilized in treatment decisions and prediction of prognosis [2, 3, 5-12]. Other parameters, namely DNA ploidy, cell proliferation, and/or angiogenesis, have also been used in assessment of prognosis [2, 3, 6, 7]. About 40% of PC patients select radical prostatectomy (RP) and the remaining select other treatments, namely brachytherapy/external beam radiation, chemotherapy, hormone therapy, immunotherapy, and/or watchful waiting [2, 3, 7, 13, 14]. Patients selecting RP have the benefit of post-RP pathology reports and opportunity for additional treatment before relapse of the disease as indicated by clinical symptoms. Patients electing other treatments often wait for adjuvant therapy until the development of clinical symptoms. Because PC is complex and heterogeneous, treatment selection based on limited pre-treatment clinical data is often inaccurate since some patients of a given Gleason grade develop aggressive disease and die within 5 years while others survive 10 years or longer [6, 11, 14, 15]. The inability to select aggressive cancers within a single Gleason grade greatly affects survival and quality of life of patients [2, 5].

Development of aggressive cancer requires that cancer cell and/or stromal cells degrade basement membrane (BM) and extracellular matrix (ECM) proteins prior to their migration to prostatic stroma [16]. This requires participation of proteases, including cysteine proteases, for invasion of cancer cells to other compartments [17, 18]. Since protease activities are balanced by the activities of their endogenous inhibitors, concurrent studies are required to define the balance of protease activity in cancer cells and their ability to invade other tissue compartments. Our objective was to assess the distribution of cathepsin B (CB) and its endogenous inhibitor stefin (cystatin) A (SA) in formalin-fixed, paraffin-embedded biopsy and prostatectomy tissue sections using immunohistochemical (IHC) methods and quantitative image analysis. Localization of

these markers in prostatectomy tissue samples from the same African American PC patients would provide an index of reliability required for the assessment of markers.

Materials and Methods

We collected a total of 30 prostate cancer cases of which we received 22 prostate needle biopsy and 22 RP tissue samples from the same African American men (8 cases had only biopsy or prostatectomy) at the Minneapolis VA Medical Center after obtaining approval from the VA and the University of Minnesota IRBs (Institutional Review Board), Minneapolis, MN. Formalin-fixed, paraffin-embedded biopsy and RP tissue sections (about 5 m thick) were obtained from the surgical pathology laboratory in addition to hematoxylin and eosin (H & E) stained sections. We collected pre-treatment and post-RP treatment clinical data, including surgery date, pre-and post-RP serum total PSA levels, clinical stage, margin/capsule status, seminal vesicle status, lymph node and/or metastasis data (Tables 1, 2). All tissue sections were graded according to the Gleason grading system [9, 19] by one of us (SLE). Biopsies and RP samples showing benign prostatic hyperplasia (BPH) or benign glandular areas were used as controls.

Antibodies Against Cathepsin B and Stefin A: Monoclonal and polyclonal antibody immunoglobulin G (IgG), namely mouse anti-human CB (clone IM27L, Oncogene Research Products, Calbiochem, Cambridge, MA) and goat anti-human SA (R& D Systems, Minneapolis, MN), used in the study were affinity purified on immobilized protein A or human SA by the manufacturers. We have already reported the molecular weights of CB (21 to 31 kDa) and SA (11 kDa) in prostatic tissues [20] [21, 22]. Our antibodies did not show any cross reactivity with other proteins in western blots [20] [21, 22]. Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO).

Immunohistochemical Localization of Cathepsin B and Stefin A: We localized CB and SA in biopsy and RP tissue sections using IHC techniques [20, 23, 24]. Briefly, antigen retrieval was carried out in 10 mM citrate buffer (pH 6.0) using a Decloaking Chamber Pro machine (Biocare Medical, Walnut Creek, CA). Mouse anti-CB antibody and goat anti-human SA were localized in adjacent sections. Since the number of biopsy sections was limited to five, we used prostatectomy sections for negative controls which were incubated with pre-immune

mouse or goat serum in lieu of the primary antibodies. The reaction products were developed, usually less than 10 min, with fresh-filtered 3, 3'-diaminobenzidine (DAB) solution (0.25 mg/ml; Sigma) in phosphate buffered saline (PBS) with 0.01% H_2O_2 as the substrate. Chromogenic reaction product was enhanced with diluted osmium tetroxide solution.

Quantification of Localization Data by Metamorph Image Analysis System: Immunostaining for CB and SA were quantified using a computer-based image analysis system equipped with Metamorph software (Universal Imaging Corp., West Chester, PA), as reported by us [20-22]. Briefly, images of CB and SA reaction products in biopsy sections were acquired at a magnification of 400X directly from the microscope slides to a computer using a digital camera (Photometrics, Tucson, AZ) attached to a Zeiss microscope. Images of CB and SA reaction products in prostatectomy sections were acquired at 200X. On the basis of gray values ranging from 4095 to 0, white to black respectively, threshold boundaries of immunostaining were created. All immunostained objects included within the designated gray value range were expressed as a percentage of the total field area under view at the selected magnification. Measurements of CB and SA are presented in range and mean with standard error of the mean (SEM),

Data Analysis: Data were analyzed using univariate and multivariate techniques. Statistical significance was determined using Student's t-test ($p<0.05$).

Results

Profile of Prostate Cancer Patients: The age of patients at prostatectomy ranged between 48 and 74 (mean 62.24±1.31). The distribution of Gleason histologic scores ranged from 6 to 8 in biopsy and prostatectomy cases (Tables 1, 2). In biopsy patients, pre-surgery serum total PSA levels ranged from 0.92 to 22.50 (mean 7.46±1.34 SEM), with no PSA data in 6 patients (Table 1). In 15/28 (53.57%) prostatectomy cases, post-surgery serum total PSA levels were >0.2ng/ml indicating biochemical failure and the remaining 13/28 (46.43%) had PSA levels of $\leq$0.2ng/ml. Clinical stages ranged from normal to T3a, including unknown stages in 14 biopsy samples (Table 1). Clinical stages ranged from T2a to T3c, N1-N-3 and unknown in prostatectomy cases (Table 2). We defined aggressive PC by the presence of cancer cells in seminal vesicles and/or pelvic lymph nodes. These characteristics of cancer cells were applied to our analysis of markers (Tables 2, 4). Post-prostatectomy

data showed 28.57% (8/28) had developed aggressive prostate cancer, 50% (14/28) had not developed aggressive cancer and aggressiveness was unknown in 21.43% (6/28) of cases (Table 2).

Immunostaining of Cathepsin B and Stefin A in BPH Glands: CB and SA protein immunostaining were present predominantly in basal cells and some cuboidal/columnar cells of BPH glands of both biopsy and prostatectomy tissue sections. Immunostaining for CB alone and SA were similar or had higher SA in biopsy and prostatectomy BPH cases (Fig. 1a, b).

Immunostaining of Cathepsin B and Stefin A in Prostate Cancer: Immunostaining of CB and SA proteins were observed in cuboidal/columnar and isolated cancer cells in biopsy tissue sections (Fig.3). The distribution of reaction products in CB alone and SA alone showed variations between and within Gleason score tumors in biopsy and prostatectomy samples (Fig. 3). In contrast, SA alone was lower than CB in biopsy and prostatectomy cases of Gleason score 6 tumors (Fig. 1a). Stefin A alone was lower in biopsy when compared to CB in cancer and essentially similar in prostatectomy samples of Gleason score 7 tumors (Fig. 1b). Ratios of CB to SA in biopsy cases were significantly elevated in Gleason score 6 ($p=0.02$) and score 7 ($p=0.004$) tumors (Fig. 2a, b). In contrast, ratios of CB to SA in BPH and cancer of Gleason score 6 prostatectomy samples were not significant ($p=0.30$) when compared to BPH (Fig. 2a), but CB to SA ratios were significant ($p=0.05$) in Gleason score 7 prostatectomy samples (Fig. 2b).

Analysis of Cathepsin B and Stefin A in BPH and Cancer: Since BPH is not an invasive tumor, it was used as a control for comparing with malignant tumors. Using our criteria of defining aggressive prostate cancer, we found 8 aggressive cancer, 14 non-aggressive cancer and status of 6 unknown in prostatectomy cases (Table 2). CB to SA ratios that did not follow the immunostaining patterns were considered as outliers in both biopsy and prostatectomy cases (Tables 3, 4). These cases are planned to be repeated with a new set of sections.

Discussion

Our interim analysis showed that the number of biopsy and prostatectomy samples from the same African American PC patients require evaluation of additional samples which are currently being added for a definitive study. Our premise is that measurements of CB and SA alone, and their ratios, would identify aggressive PC in biopsy samples as they did in prostatectomy cases reported earlier [20, 25]. Our immunostaining data indicated inconsistent patterns in biopsy and prostatectomy samples. We also found that small tissue section size contributed to the reaction products due to the edges of biopsy sections. That problem was not associated with prostatectomy samples. We hope to submit our manuscript with additional data this summer.

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Table 1. Distribution of Gleason Histological Scores in Needle Biopsies

Gleason Score	6	7	8	Total
[1]Number of Samples	7	14	3	24
[2]PSA, Mean±SEM	5.95±1.07	8.42±2.09	-	7.46±1.34
Range	1.40 - 9.18	0.92 - 22.50	-	0.92 - 22.50
No PSA data	-	3	3	6 (25.00%)
[3]TNM (Jewett-Whitmore)				
Normal	1	-	-	1 (4.17%)
T2c (B2, B3)	1	3	1	5 (20.83%)
T3a (C1)	1	3	-	4 (16.67%)
Unknown	4	8	2	14 (58.33%)

SEM = Standard Error of the Mean

[1]Twelve cases were not included because of unavailable slides or cancer in sections.

[2]Pre-RP surgery serum total PSA levels.

[3] TNM and Jewett Whitmore classifications are cited from Ellis WJ, and Lange PH. Prostate Cancer. Endocrinol Metab Clin North Am., 1994; 23:809-824.

Table 2. Distribution of Gleason Histological Scores After Radical Prostatectomies of African American Patients

Gleason Score	6	7	8	Total
[1]Number of samples	16	11	1	28
Range of age at surgery	48 - 74	54 - 70	58	48 - 74
Mean±SEM	62.92±2.01	61.62±1.66	-	62.24±1.31
[2]Serum PSA levels > 0.2ng/ml	6	8	1	15 (53.57%)
Serum PSA levels ≤ 0.2ng/ml	10	3	-	13 (46.43%)
TNM (Jewett-Whitmore)				
T2a - T2c (B1, B2, B3)	9	2	-	11 (39.29%)
T3a - T3c (C1, C2)	4	3	-	7 (25.00%)
N1 - N3 (D1)	-	1	1	2 (7.14%)
Unknown	3	5	-	8 (28.57%)
[3]Aggressive PC	1	6	1	8 (28.57%)
Non-Aggressive PC	10	4	-	14 (50.00%)
Unknown status	5	1	-	6 (21.43%)

PC = Prostate Adenocarcinoma

[1]Eight cases were not included because of unavailable slides or cancer in sections.

[2]PSA > 0.2ng/ml indicated biochemical failure.

[3]Aggressive PC was defined by the presence of cancer cells in seminal vesicle and/or pelvic lymph nodes.

Table 3. Immunohistochemical Distribution of Cathepsin B, Stefin A, and Their Ratios in Gleason Score 6-8 Biopsies and Prostatectomies

	# of Cases	CB (Range)	SA (Range)	CB/SA Ratio (Range)
Biopsy				
[1]BPH	15	0.78 ± 0.10 (0.33-1.64)	1.38 ± 0.19 (0.35-2.89)	0.86 ± 0.20 (0.19-3.08)
[2]Gleason 6	3	0.72 ± 0.20 (0.37-1.07)	0.17 ± 0.05 (0.06-0.24)	4.59 ± 0.62 (3.60-5.74)
Gleason 7	13	0.57 ± 0.05 (0.26-0.91)	0.28 ± 0.05 (0.08-0.62)	3.53 ± 0.81 (0.62-10.41)
Gleason 8	3	0.65 ± 0.10 (0.48-0.83)	0.55 ± 0.24 (0.19-1.01)	1.75 ± 0.83 (0.81-3.40)
Prostatectomy				
[3]BPH	23	0.43 ± 0.06 (0.11-1.12)	0.91 ± 0.17 (0.11-2.57)	0.92 ± 0.17 (0.10-3.28)
[4]Gleason 6	10	0.26 ± 0.04 (0.06-0.52)	0.17 ± 0.03 (0.04-0.37)	1.56 ± 0.16 (0.91-2.56)
[5]Gleason 7	9	0.28± 0.06 (0.10-0.62)	0.29 ± 0.08 (0.05-0.65)	1.70 ± 0.50 (0.35-4.91)
Gleason 8	1	0.13	0.25	0.51

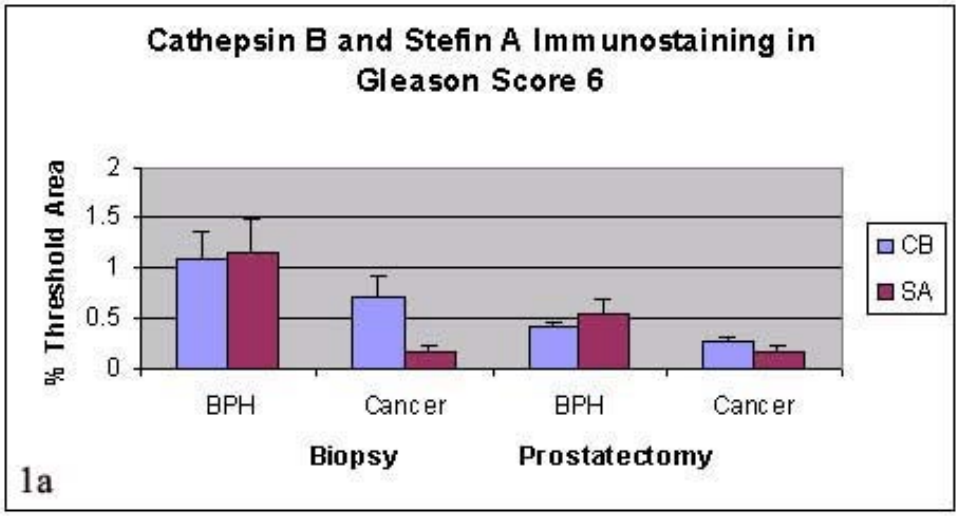
[1]Two BPH biopsy cases were considered outliers due to high CB immunostaining or high CB to stefin A ratio. The outliers did not follow patterns of immunostaining found in other cases.
[2]Three Gleason score 6 biopsy cases were considered outliers due to high CB or stefin A immunostaining, or high CB to stefin A ratio.
[3]One BPH prostatectomy case was considered an outlier due to high CB to stefin A ratio.
[4]Six Gleason score 6 prostatectomy cases were considered outliers due to high CB immunostaining, stefin A immunostaining and/or CB to stefin A ratio.
[5]Two Gleason score 7 prostatectomy cases were considered outliers due to high CB or stefin A immunostaining.

Table 4. Distribution of Cathepsin B to Stefin A Ratios in Aggressive PC Biopsies

PC Patients	Biopsy - CB/SA Ratio # of patients (mean ± SEM)	Prostatectomy - CB/SA Ratio # of patients (mean ± SEM)
[1]Aggressive	6 (1.79 ± 0.58)	7 (1.64 ± 0.58)
Non-Aggressive	10 (4.52 ± 0.91)	10 (1.56 ± 0.27)
Unknown	8 *	11 **

* Includes four outliers and four unknown aggressiveness
** Includes five outliers and six unknown aggressiveness

[1]Aggressive PC was defined by cancer cell positive seminal vesicles and/or pelvic lymph nodes.



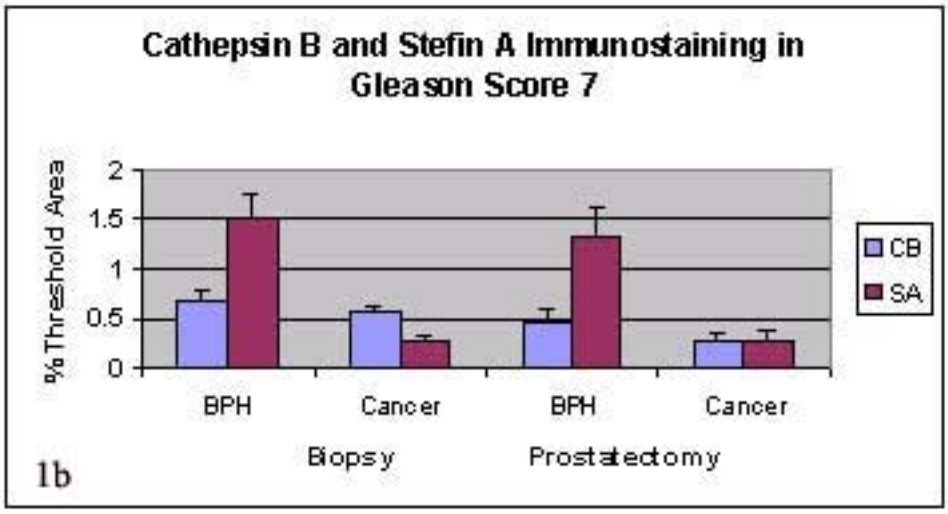
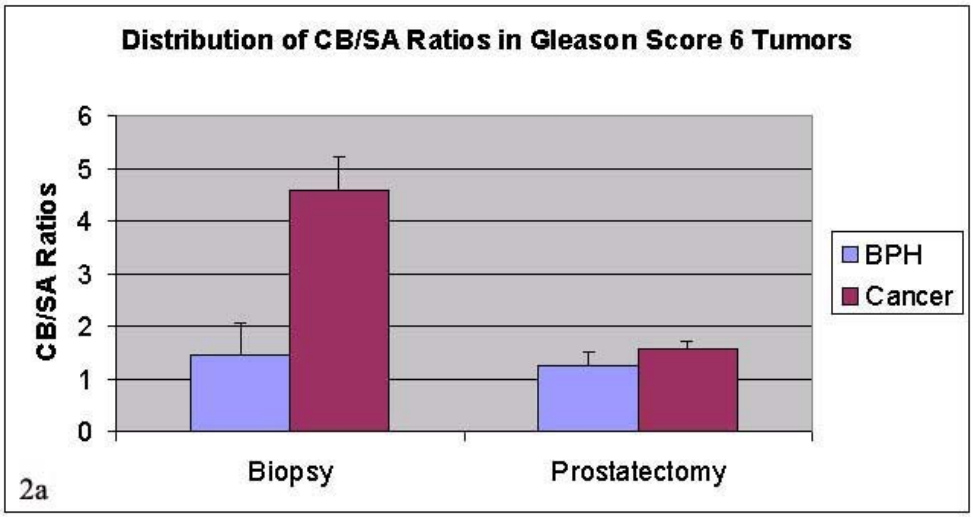


Figure 1a shows that CB alone and SA alone were similar in biopsy and prostatectomy sections of BPH. Stefin A was lower in biopsy and prostatectomy sections when compared to CB in Gleason score 6 cancer.

Figure 1b shows that SA alone is higher in comparison to CB in biopsy and prostatectomy sections of BPH. Stefin A alone was lower in cancer when compared to CB alone in Gleason score 7 biopsies. However, CB alone and SA alone were similar in prostatectomy samples of Gleason score 7 tumors.



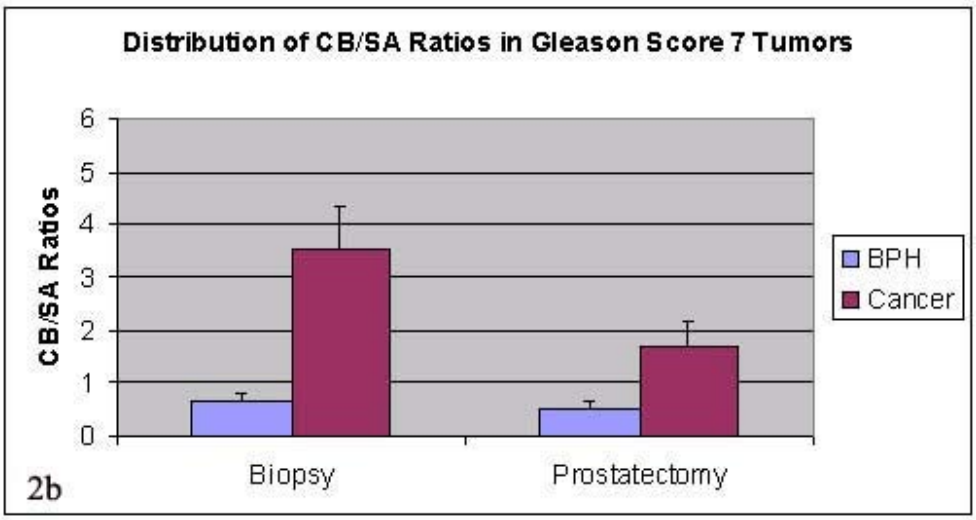


Figure 2a and b show CB to SA ratios in cancer that were significantly elevated in Gleason score 6 (p=0.02) and Gleason score 7 (p=0.004) biopsy samples when compared to BPH. CB to SA ratios in BPH and cancer of Gleason score 6 prostatectomy sections were not significant (p=0.30), however the ratios were significant in Gleason score 7 prostatectomies (p=0.05). CB to SA ratios in biopsy were significantly higher than prostatectomy samples in Gleason score 6 cancer (p=0.04). The same ratios were not significant in Gleason score 7 cancer (p=0.07).

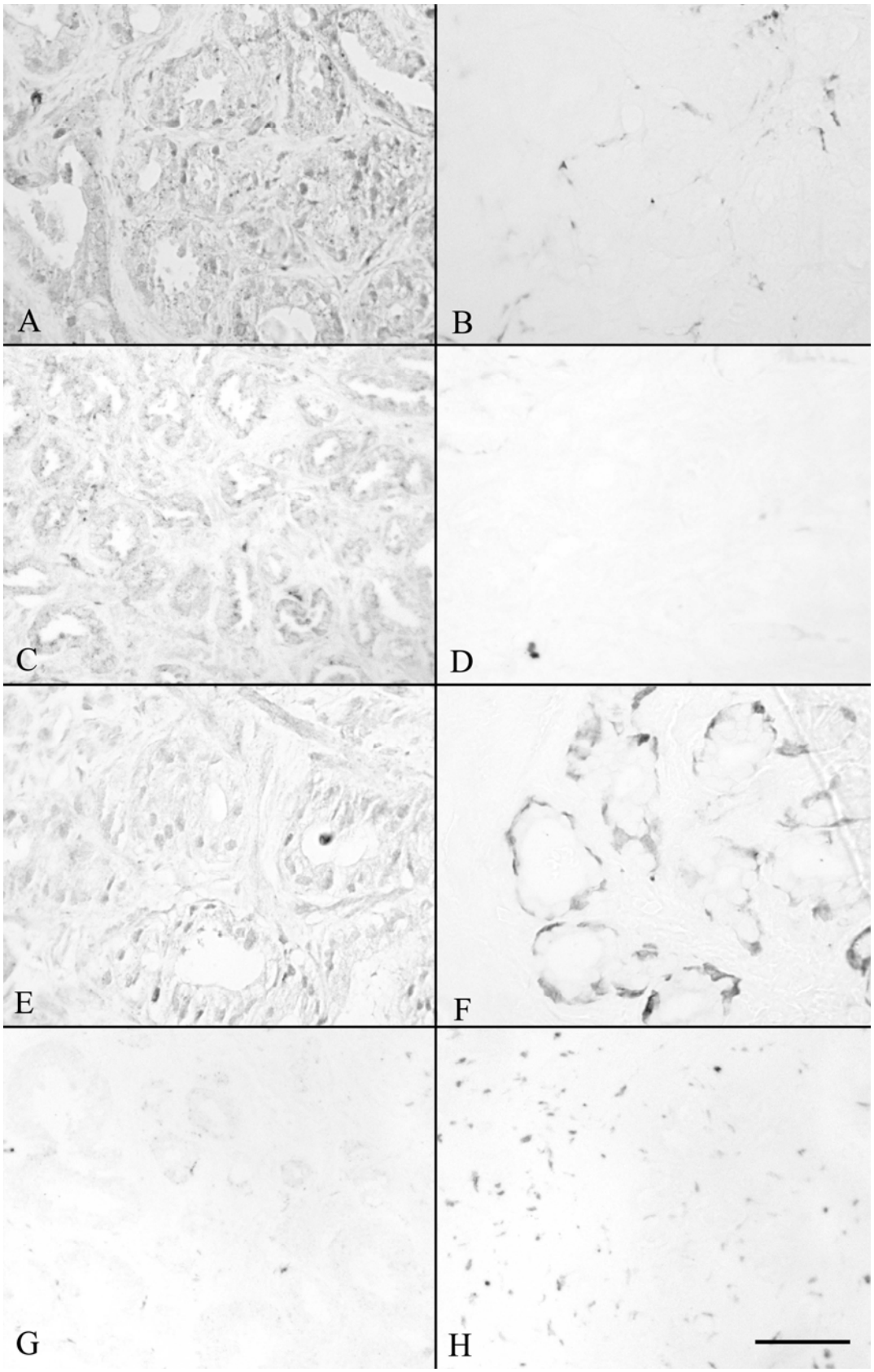


Figure 3

Figure 3. Immunohistochemical Localization of Cathepsin B and Stefin A in African American Biopsies and Prostatectomies

A. Micrograph shows elevated CB immunostaining in cuboidal/columnar and isolated cancer cells in a Gleason score 6 biopsy.

B. Micrograph illustrates decreased SA in a Gleason score 6 biopsy from the same patient in figure 3A. The CB to SA ratio was 3.87.

C. Micrograph illustrates a Gleason score 6 prostatectomy with elevated CB immunostaining.

D. Immunostaining for SA is low in a Gleason score 6 prostatectomy from the same patient in figure 3C. The CB to SA ratio was 10.39.

E. Immunostaining of CB in cancer cells in a Gleason score 7 biopsy of an African American. The CB levels are lower in comparison to figure 3A.

F. Micrograph illustrates a Gleason score 7 biopsy with SA immunostaining from the same patient in figure 3E. The levels are elevated when compared to figure 3B. The CB to SA ratio was 0.19.

G. Immunostaining for CB in a Gleason score 7 prostatectomy decreased when compared to figure 3C.

H. Micrograph shows a Gleason score 7 prostatectomy, from the same patient in figure 3G, with elevated SA immunostaining when compared to figure 3D. The CB to SA ratio was 0.21. Bar illustrates magnification of images. It is equal to 50μm in prostatectomies and 100μm in biopsies.